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- Identification of tributyrin in ButyraGen™ by HPTLC PRO
- Analysis of Brazilian medicinal plants by HPTLC PRO
- HPTLC fingerprinting of Ashwagandha formulations

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CAMAG®

CAMAG (Switzerland)
Sonnenmattstrasse 11 • 4132 Muttenz
Phone +41 61 4673434 • Fax +41 61 4610702
info@camag.com • www.camag.com

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HPTLC method for the identification of tributyrin in ButyraGen™

Left to right: Wilmer H. Perera, Ph.D., David Bom, Ph.D., Mallory Goggans, M.S. and Michael Lelah, Ph.D.

Dr. Wilmer H. Perera is the Lab Manager at CAMAG Scientific, Inc. in Wilmington, NC and he is dedicated to the development of HPTLC methodologies that can be applied to the dietary supplement, food and cosmetic industries, and more to come. Dr. Michael Lelah and Mallory Goggans are with NutriScience Innovations, a dietary ingredient development and distribution company with headquarters in Milford, CT. Dr. David Bom is a consultant for NutriScience. NutriScience is the developer of ButyraGen™.

Introduction

ButyraGen™ is a new dietary ingredient, a prebiotic direct butyrate generator [1]. The primary active ingredient, tributyrin (glycerol with three butyrate arms), is hydrolyzed in the body to the short chain fatty acid butyrate (butyric acid). Butyrate is a postbiotic involved in supporting digestive health through reducing gut permeability and also is an important gut signaling molecule for the gut-brain axis and other organ support [2]. Although tributyrin itself is an oil, ButyraGen™ is a spray-dried powder. This makes ButyraGen™ a hybrid – the material is a powder but it contains an oil.

Dietary ingredients for use in dietary supplements manufactured under cGMP, require testing for identity, purity, strength and composition [3]. The identity test can also be used as a test for adulteration, which is a general concern for dietary ingredients and supplements. The identity test can help confirm whether an ingredient has been adulterated.

HPTLC is widely used in the dietary supplement industry for the identification of botanicals, botanical concentrates and botanical extracts. The purpose of this study was to develop an HPTLC method for the identification of ButyraGen™ using the identification of tributyrin, the main active ingredient in ButyraGen™ (> 50% content) as the primary identification marker. The suitability of the method for this purpose was determined using tributyrin as a standard and also by comparing it against other fatty acids and lipids. Suitability is fit for purpose, which is the appropriate standard for the development of an identification method for a dietary ingredient [4]. Commonly used and

inexpensive food fatty acids and oils are compared to determine if the method is sufficiently sensitive and specific to distinguish ButyraGen™ and tributyrin from these materials, which may be considered potential adulterants. Additionally, a negative control consisting of the other components of ButyraGen™ (without tributyrin) was evaluated to determine the effect of these other components in the product.

The use of HPTLC for the identification of oils is far less well known although methods for the determination of fatty oils have been developed [5]. Many manufacturers of dietary ingredients and dietary supplements have HPTLC instrumentation in their analytical labs and conduct identity testing of botanicals on a regular basis. Thus, HPTLC is an ideal method to identify tributyrin in ButyraGen™ but it can be used for many other applications. The HPTLC PRO System boosts the applicability of the technique since it is a fully automated system where multiple samples can be analyzed in sequence, overcoming the environmental effects produced by the previous open system. HPTLC PRO also adds a more rigorous control of the gas phase and although still under development as an analytical tool, it will become a standard and powerful technique for advanced research and quality control purposes.

Standard solutions

4.0 mg of tributyrin and triacetin are dissolved in 1.0 mL of methanol. The Universal HPTLC Mix (UHM) solution was prepared as described in literature [6] and used as system suitability test (SST).

Sample preparation

ButyraGen™ and ButyraGen™ placebo (ButyraGen™ without the primary active tributyrin), glycerol monostearate, raw cocoa butter and palm kernel oil were prepared at 10.0 mg/mL methanol. 20.0 µL of medium chain triglycerides and linseed oil were dissolved in 980.0 µL of methanol and toluene, respectively. Samples were sonicated for 10 min at room temperature and centrifuged at 3000 rpm for 5 min as needed. The supernatant was used for further analysis.

Chromatogram layer

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

Sample application

10.0 µL of ButyraGen™ and ButyraGen™ placebo, glycerol monostearate, raw cocoa butter solutions, 2.0 µL of medium chain triglycerides, palm kernel oil and linseed oil solution while 40.0 µL and 20.0 µL of triacetin and tributyrin solutions, respectively, are applied as bands with the HPTLC PRO Module APPLICATION, 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. The first rinsing step (bottle 1 solvent) is done with methanol – acetonitrile – iso-propanol – water – formic acid 250:250:250:250:1 (*V/V*) and the second rinsing step (bottle 2 solvent) is done with methanol – water 7:3 (*V/V*).

Chromatography

Plates are prepared in the HPTLC PRO Module DEVELOPMENT before development by pre-drying for 30 s, activation at 33 % relative humidity for 10 min using a saturated solution of magnesium chloride, conditioning with hexane – ethyl acetate 17:3 (*V/V*) at a pump power of 40 %, development with hexane – ethyl acetate 17:3 (*V/V*) to the migration distance of 70 mm (from the lower edge), followed by drying for 5 min.

Post-chromatographic derivatization

The plate was immersed into primuline (0.05 % in acetone – water, 4:1(*V/V*)) using the Chromatogram Immersion Device 3, immersion speed 3 cm/s and immersion time 5 s, dried for 5 min with cold air.

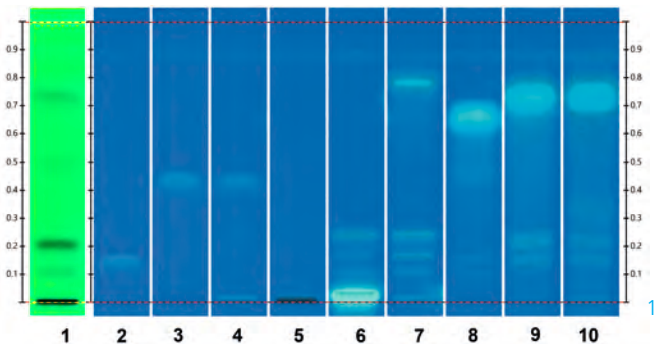
Documentation

Images of the plate are captured with the TLC Visualizer 2 in UV 254 nm prior to derivatization and in UV 366 nm after derivatization.

Results and discussion

The HPTLC analysis was qualified by using an UHM as system suitability test [6]. Three main quenching zones were observed in short wavelength UV 254 nm for the SST with R_F 0.11 ± 0.04, 0.21 ± 0.04 and 0.76 ± 0.04 in the figure below. The results are quite straightforward, ButyraGen™ (track 4) is identified by the tributyrin reference

standard (track 3) and none of the other fatty acids tested should moved to this position. The other materials tested represent a range of food and other fatty acids which potentially could be used as adulterants to replace tributyrin in ButyraGen™. Tributyrin is a triglyceride with a glycerol backbone and three butyrate side chains. Triacetin is a triglyceride with a glycerol backbone and three acetate side chains. Glycerol monostearate is a long chain monoglyceride commonly used as a food emulsifier. The main constituent in cocoa butter is the triglyceride derived from palmitic, oleic and stearic acid. Cocoa butter also contains other unsaturated and saturated fatty acids. Medium chain triglycerides are triglycerides with two or three medium chain fatty acids. Palm kernel oil is high in saturated fats and lauric acid. Linseed oil (also known as flax seed oil) is high in unsaturated diglycerides and triglycerides, including alpha-linoleic acid.



HPTLC analysis of the UHM (track 1) in UV 254, triacetin and tributyrin (tracks 2 and 3), ButyraGen™ and ButyraGen™ placebo (tracks 4 and 5), glycerol monosterate, raw cocoa butter, medium chain triglycerides, palm kernel oil and linseed oil (tracks 6–10) in UV 366 nm post derivatization with primuline solution.

This method of HPTLC chromatographic separation is very specific for the different types of mono-, di-, and triglycerides indicating very good specificity for tributyrin and ButyraGen™. These results indicate the suitability (fit for purpose) of the method for the identification of tributyrin and ButyraGen™. Certainly, for the wide range of pure and naturally occurring complex fatty acid esters tested here, ButyraGen™ and tributyrin are completely and specifically distinguished. In the event that ButyraGen™ was to be adulterated with any of these products, this identity test method will be able to confirm the presence of such an adulterant. This indicates the method as suitable for confirming the presence of a variety of potential adulterants.

[1] https://nutriscienceusa.com/product/butyragen

[2] Canani R.B. *et al.* World J Gastroenterol 17(12) (2011) 1519-1528.

[3] FDA, Code of Federal Regulations, 21CFR111.70. https://www.accessdata.fda.gov/scripts/cdrh/cfdocs/cfcfr/CFRSearch.cfm?fr=111.70&SearchTerm=identity

[4 Wenclawiak B. *et al.* Quality Assurance in Analytical Chemistry (2010) 215-245.

[5] Identification of fixed oils, HPTLC Association https://www.hptlc-association.org/methods/methods_for_identification_of_herbals.cfm

[6] Do T.K.T. *et al.* J Chromatogr A 1638 (2021) 461830.

Further information is available on request from the authors.

Contact: Dr. Wilmer H. Perera, CAMAG Scientific, Inc., 515 Cornelius Harnett Drive Wilmington, NC 28401, USA, wilmer.perera@camag.com

Dr. Michael Lelah, NutriScience Innovations, 130 Old Gate Lane, Unit C, Milford, CT 06460, mlelah@nutriscienceusa.com

Discrimination of *Monteverdia ilicifolia* leaf from its adulterants by HPTLC PRO

Dr. Jane Manfron and Dr. Wilmer H. Perera

Dr. Wilmer H. Perera serves as Lab Manager at CAMAG Scientific, Inc. in Wilmington, NC. He has collaborated closely with Dr. Jane Manfron's group in the development of HPTLC methods for the quality control of Brazilian herbal drugs. Herein, he transferred the regular HPTLC method for discriminating *Monteverdia ilicifolia* from adulterants into an HPTLC PRO method to improve the efficiency of the process.

Dr. Jane Manfron holds the position of Associate Professor at the State University of Ponta Grossa, Brazil, and leads the Pharmacognosy Laboratory. Her research focuses on the identification of plant species, using morphology and microscopy. In addition, she studies the chemistry and biological effects of essential oils. Her team involved in this research are Kevin A. Antunes, MSc., Luciane M. Monteiro, MSc., Vera L.P. dos Santos, Ph.D., and collaborators Gustavo Heiden, Ph.D. and Ernestino de S. G.G, Ph.D. from Embrapa Clima Temperado, Pelotas, Rio Grande do Sul, Brazil.

Introduction

The species *Monteverdia ilicifolia* (Mart. ex Reissek) Biral, also known as espinheira santa, is one of the most commercialized species in Paraná State, Brazil. It is widely used to treat gastritis and gastric ulcers. Despite its popularity, the market is plagued by issues of low-quality products. Common adulterants include *Citronella gongonha* (Mart.) R.A. Howard, *Jodina rhombifolia* (Hook. & Arn.) Reissek, *Sorocea bonplandii* (Baill.) W.C. Burger *et al.*, *Zollernia ilicifolia* (Brongn.) and M*onteverdia aquifolia* [1]. Studies have shown that several herbal samples sold in the market have been misidentified and *M. aquifolia* is frequently sold as *M. ilicifolia* [1].

An HPTLC PRO method based on the fingerprint of flavonoids and phenolic acids was developed for the discrimination of all relevant herbal drugs and applied for the analysis of commercial samples.

HPTLC is considered the gold standard for the identification of botanicals in many countries. The fully automated version of the technique, HPTLC PRO, brings more hands off during the analysis. The capabiliy to regulate the gas phase during chromatography, using the HPTLC PRO Module DEVELOPMENT, improves the separation efficiency when compared to conventional HPTLC performed by development in the ADC 2.

Standard solutions

Epicatechin was prepared at 28 µg/mL, quercetin and chlorogenic acid at 200 µg/mL, and rutin at 400 µg/mL in methanol. The Universal HPTLC mixture (UHM; prepared in house) was used as a system suitability test (SST).

Sample preparation

5.0 g of leaves of *Monteverdia ilicifolia*, adulterants and herbal products were milled and extracted with 50.0 mL of methanol by sonication for 15 min at room temperature. The solution was filtered over cotton and then dried using a rotatory evaporator to afford 10 mg of the extract. The evaporated extract was dissolved in a suitable amount of methanol to yield 10 mg/mL.

Chromatogram layer

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

Sample application

5.0 µL of sample and standard solutions, and 2.0 µL of the UHM are applied as bands with the HPTLC PRO Module APPLICATION, 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. The first rinsing step (bottle 1 solvent) is done with methanol – ace-

tonitrile – isopropanol – water – formic acid 250:250:250:250:1 (*V/V*) and the second rinsing step (bottle 2 solvent) is done with methanol – water 7:3 (*V/V*).

Chromatography

The HPTLC PRO Module DEVELOPMENT used the following parameters: pre-drying for 30 s, activation at 33 % relative humidity for 10 min using a saturated solution of magnesium chloride, conditioning with *n*-butyl acetate – methanol – water – formic acid 15:4:2:2 (*V/V*) at a pump power of 25 % from 50 to 70 mm developing distance, development with *n*-butyl acetate – methanol – water – formic acid 15:4:2:2 (*V/V*) to the migration distance of 70 mm (from the lower edge), followed by drying for 5 min [2].

Post-chromatographic derivatization

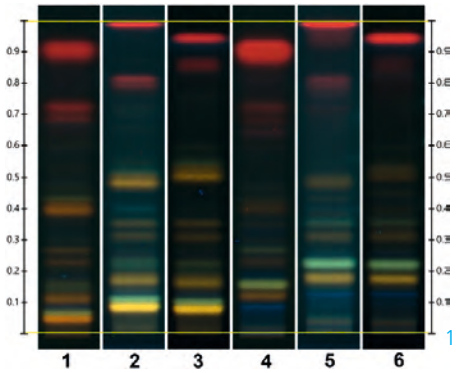
Plates are derivatized using the HPTLC PRO Module DERIVATIZATION by heating the plate at 100 °C for 180 s then spraying 1.5 mL of Natural Product reagent (NP) (1,0 g of 2-aminoethyl diphenylborinate in 100 mL of methanol) under reduced pressure with nozzle 1 at spraying level 3. A second derivatization was performed by using 1.8 mL of anisaldehyde reagent (add slowly 10.0 mL of acetic acid and 5.0 mL of sulfuric acid to 85.0 mL of ice-cooled methanol, mix, cool to room temperature, add 0.5 mL of *p*-anisaldehyde) under reduced pressure with nozzle 2 at spraying level 2, then the plate is heated at 100 °C for 90 s.

Documentation

Images of the plate are captured with the TLC Visualizer 2 in UV 254 nm after development, UV 366 nm after derivatization with NP reagent, and white light after subsequent derivatization with anisaldehyde reagent.

Results and discussion

The fingerprint obtained from *Monteverdia ilicifolia* and related species in the ADC 2 is compared to the fingerprint obtained from the HPTLC PRO System as shown in the figure below. The R_F position of all zones obtained with the HPTLC PRO System appears to be higher than with the ADC 2. When no preconditioning or conditioning is used, the chlorophylls migrate with the solvent front but with conditioning all zones are observed between the application position and the solvent front. Using the HPTLC PRO System a slight separation improvement is observed in the lower part of the chromatogram, below R_F 0.4.

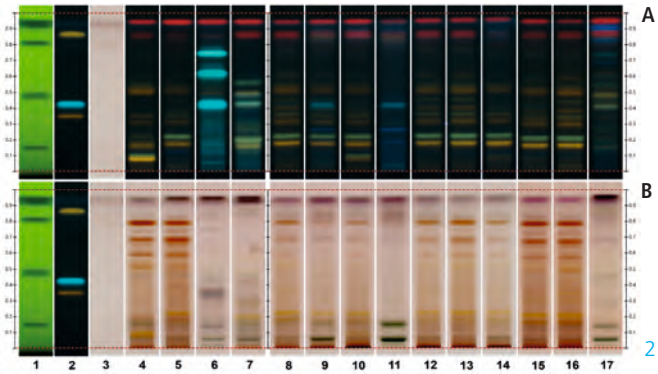


Chromatograms of Monteverdia ilicifolia and Monteverdia aquifolia in the ADC 2 (tracks 1 and 4 respectively), and the HPTLC PRO System with no preconditioning or conditioning (tracks 2 and 5) and with conditioning at a pump power of 25 % from 50 to 70 mm developing distance (tracks 3 and 6).

Using the HPTLC PRO System with conditioning, the fingerprint of *M. ilicifolia* is different from those of the potential adulterants including the related species, considering both detection modes. UV 366 nm after derivatization with NP reagent is the most suitable detection mode for identification. In the SST, the UHM generates three main quenching zones in shortwave UV (254 nm) at R_F 0.15±0.01, 0.47±0.01, and 0.82±0.01.

Nine samples purchased on the Brazilian market and one online in the USA were analyzed using the developed method. The fingerprints of the samples on tracks 8, 12, 13, 15 and 16, matched that of the *M. aquifolia* BRM on track 5. The sample on track 9 also displayed a fingerprint of *M. aquifolia*, but with a couple of additional blueish zones at R_F 0.26 and 0.42, indicating the presence of adulterants. The sample on track 11 exhibited a distinct fingerprint, including these blueish zones. This fingerprint had been previously identified as *Sorocea bonplandii* [1] implying that the sample on track 9 with a similar *M. aquifolia* fingerprint has also been adulterated with *S. bonplandii*. The sample on track 10 revealed a clear fingerprint similar to that of the *M. ilicifolia* BRM while the sample on track 14 also shows similarities to *M. ilicifolia* with a fainter zone of the flavonoid polyglycosides at R_F ~0.09. The sample on track 17 exhibited a few zones characteristic of *Jodina*

rhombifolia, although other zones not associated with any adulterants are also observed. Only two of the ten samples match the fingerprint of *M. ilicifolia*. Although *M. ilicifolia* and *M. aquifolia* thrive in different habitats, the misuse of *M. aquifolia* for *M. ilicifolia* in commercial samples is very common. HPTLC PRO proves to be an effective approach to rapidly discriminate *M. ilicifolia* from its adulterants in commercialized samples using the flavonoid fingerprint.



2

*Chromatograms of the UHM in UV 254 nm (track 1), rutin, chlorogenic acid, and quercetin in UV 366 nm after derivatization with NP reagent (track 2, with increasing R_F), epicatechin 28 µg/mL post derivatization with anisaldehyde on top of NP reagent (track 3), Monteverdia ilicifolia botanical reference material (BRM) (track 4), Monteverdia aquifolia BRM (track 5), Citronella gorgonha BRM (track 6), Jodina rhombifolia BRM (track 7) and herbal products tested from Brazilian market (tracks 8–16) and from USA (track 17). BRMs and samples in longwave UV after derivatization with NP reagent (**A**) and in white light after derivatization with NP + AS (**B**).*

[1] Antunes KA *et al.* Microsc Microanal (2023) DOI: 10.1093/micmic/ozad098

[2] Antunes KA *et al.* Nat Prod Res, accepted (2023)

Further information is available on request from the authors.

Contact: Dr. Jane Manfron, Postgraduate Program in Pharmaceutical Sciences, State University of Ponta Grossa, 4748 Carlos Cavalcanti Avenue, 84030-900 Ponta Grossa, PR, Brazil. janemanfron@hotmail.com

Dr. Wilmer H. Perera, CAMAG Scientific, Inc., 515 Cornelius Harnett Drive Wilmington, NC 28401, USA, wilmer.perera@camag.com

CAMAG® TLC Visualizer 3

The TLC Visualizer 3 stands as a top-tier imaging and documentation system, delivering unmatched performance across white light, long-wave UV (366 nm), and short-wave UV (254 nm) for uniformly brilliant illumination. Engineered to capture premium-quality images, this documentation system is equipped with an advanced industrial camera featuring the latest CMOS image sensor technology.

Powered by the *visionCATS* HPTLC software, the TLC Visualizer 3 excels at detecting even the most subtle zones. Specifically designed to support the HPTLC workflow, this best-in-class software offers an array of sophisticated image enhancement tools, fully harnessing the capabilities of the TLC Visualizer 3. It allows for the simultaneous display of references and samples, whether from the same or different plates, in various illumination modes, side by side.

This high-end device guarantees maximum reproducibility while maintaining compliance with cGMP/GLP and 21 CFR Part 11 standards.

For more details, please visit our website at **www.camag.com/tlcvisualizer3**.

CAMAG Scientific Inc. – History and present

CAMAG initially entered the US market through distributorships but later established CAMAG Scientific Inc. (CSI), a Swiss-owned corporation. In 1988, Dr. Dieter Jänchen hired Roger James as CSI's general manager, with an office in Wilmington, NC. Roger managed the business and primary sales responsibilities, laying the foundation for CAMAG's permanent presence in the US.

In 1992, at an International HPTLC Symposium in Raleigh, Roger met Dr. Eike Reich, a Virginia chemistry professor. This collaboration led to CSI's laboratory establishment, requiring a move to a larger location in Wilmington. Dr. Reich contributed between 1994 and 1997, working on the lab, customer courses, and botanical analyses. Key relationships were formed in the dietary supplement industry during this period. Dr. Reich moved to Switzerland in 1998, and chemist Amanda Bieber oversaw CSI's lab growth.

The 1990s witnessed staff changes, including Jean Pollard in 1999 and Don Oates in 2000. Roger, supported by various sales reps and Don, managed the business. The core team of Roger James, Don Oates, and Jean Pollard remained stable throughout the decade.

The new decade brought changes: Judith Nichols joined as lab manager in 2009, Fabien Scorza as a sales representative in 2010, and Tina Howell as office manager in 2011. In 2012, Roger James retired, and Don Oates assumed the general manager role, hiring David Hansen for the service department. In 2016, Gary Binas joined as a full-time sales rep, significantly boosting business on the West Coast.

The approaching new decade continued to bring changes: Judith left in late 2018, and Dr. Wilmer Perera was hired as lab manager. In 2020, Fabien Scorza returned as the sales manager, and Alex Hamby joined in February 2021. Sadly, Don Oates retired in April 2021, and Fabien passed away in November 2022 after battling cancer.

CAMAG Scientific, Inc. staff 2023

Tina Howell, CAMAG Scientific Inc.'s Business Manager, has 40 years of finance management experience. Her 12 years at CAMAG encompassed customer service, operations, support, human resources, and finance. She holds a B.S. in Business Administration from the University of North Carolina-Wilmington's Cameron School of Business. After 10 years in medical administration and 18 years in custom-home building, she found her home at CAMAG. Outside of work, she enjoys her growing family, including nine lively grandsons.

Dr. Wilmer Perera, CAMAG Scientific Inc.'s Laboratory Manager since February 2019, is a versatile professional. He develops HPTLC methodologies, manages analyses, and aids sales, while also offering HPTLC courses. Wilmer collaborates with local educational institutions, serves as the Secretary General for the North America Chapter of the HPTLC Association, and organizes educational webinars. He's an expert advisor for USP and a part of the Pan-American Expert Panel. Before joining CAMAG Scientific, he specialized in isolating and identifying bioactive compounds from natural sources, earning degrees in chemistry and organic chemistry with a focus on natural products. His academic journey led to a doctorate in Biochemistry.

Bruce Semkam joined CAMAG Scientific, Inc. in March 2023 as the Eastern USA Sales Representative, focusing on TLC/HPTLC product marketing and consultation. He holds a B.A. in Biology from Kean University, Elizabeth, NJ, and a Master's in Business Technology with a Digital Marketing concentration. Bruce began in pharmaceuticals and later shifted to technical sales, eventually leading him to CAMAG. He values being part of a globally renowned, innovative, and high-performance company.

Gary Binas, with 15 years of sales and leadership experience, joined CAMAG Scientific, Inc. Over the past seven years, he's developed expertise in HPTLC and DBS analysis. As the US Western region Sales Representative, he collaborates with CAMAG teammates to deliver cutting-edge HPTLC instruments to customers. He also excels in post-sales service, providing insights for clients to optimize their analytical objectives. With a cum laude Bachelor of Arts in Chemistry from the University of California, Santa Barbara, Gary enjoys family time, exploring the world, and continuous learning.

Shannon Ehlers, a Sales and Service Representative for CAMAG Scientific, Inc.'s Midwestern region since May 2023, handles sales, installations, maintenance, and repairs for CAMAG HPTLC instruments. Shannon previously worked for CAMAG from 2011–2012 and holds a BA in Chemistry from Central College in Iowa. His past roles encompass research, agriculture, and technical services with different companies.

Alex Hamby, Technical Service Representative since February 2021, oversees CAMAG instrument installations and maintenance in the Eastern US. With a Bachelor of Science degree from the University of Central Arkansas, including a minor in cognitive neuroscience, he previously worked as a Chemist II, managing lab equipment and computer systems in a production lab.

Victor Chavez, a Service Technician Representative since May 2023, covers the entire West Coast, Western Canada, and North America remotely. His primary responsibilities include instrument installations and maintenance. Victor brings experience from a robotics company, where he handled North American installations and maintenance, including advanced machine learning, coding, and instrument maintenance, largely self-taught due to his curiosity.

Inositol phosphate analysis by HPTLC

Corinna Henninger, M. Eng.

Corinna Henninger is a Ph.D. student at the Karlsruhe Institute of Technology, under supervision of Adj. Prof. Katrin Ochsenreither. Her research focuses on the enzyme class of phytases, the analysis of the obtained degradation products, and the design of novel phytases using molecular biology. Her work is conducted at the Offenburg University of Applied Sciences, under co-supervision of Prof. Thomas Eisele, an expert in the field of enzyme production.

Introduction

Phytases (IUBMB Enzyme Nomenclature: EC: 3.1.3.26) catalyze the stepwise dephosphorylation of phytate (*myo*-inositol-1,2,3,4,5,6-hexakisphosphate or $InsP_6$), the natural storage component of phosphate in plants. However, phytate shows poor digestibility in non-ruminant animals such as swine, poultry and fish due to their lack or low activity of $InsP_6$-hydrolyzing enzymes in the gastrointestinal tract. Therefore, phytases are utilized as a feed additive to release the bound phosphate. The analysis of myo-inositol phosphates ($InsP_x$) is challenging and time consuming, particularly in terms of separation and detection.

However, when dealing with a large number of samples in the screening for phytases during protein engineering, having a fast and robust analysis method is crucial to reliably identify promising novel enzymes or target variants.

Considering high sample throughput and separation of all isomeric pools as well as free phosphate, HPTLC is most suitable as a fast and inexpensive screening method. Furthermore, the utilization of an enzyme assisted post-chromatographic derivatization step makes the method highly specific for $InsP_x$.

Standard solutions

1.0 g/L phosphate (P_i, TraceCERT® for IC) in water is utilized. Inositol phosphates Ins(3)P_1 (sodium salt), Ins(2,4)P_2 (sodium salt), Ins(1,4,5)P_3 (sodium salt), Ins(2,3,5,6)P_4 (sodium salt), Ins(1,3,4,5,6)P_5 (sodium salt) Ins(1,2,3,4,5,6)P_6 (sodium salt) are dissolved in water.

Sample preparation

Phytic acid (1.66 g/L in 50 mM NaOAc pH 5.5 and 3.6) are digested enzymatically using 10 U/L phytase activity at 37 °C for 24 h. Samples are taken periodically (after 5, 30, 60, 120, 180, 240, 300 min and 24 h) and stopped by heat.

Chromatogram layer

HPTLC Cellulose F (Merck), 20 x 10 cm and 10 x 10 cm are used.

Sample application

2.0 µL of sample solutions and 2.0–19.0 µL of standard solutions are applied as bands with the Automatic TLC Sampler (ATS 4), 20 tracks, band length 6.0 mm, distance from the left edge 15 mm, track distance 10 mm, distance from the lower edge 10 mm.

Chromatography

Plates are developed in a twin through chamber after chamber saturation for 30 min with 20 mM NaOAc – 10 mM NH_4Cl – 2-propanol – 1,4-dioxane – acetic acid 500:520:200:6 (*V/V*) up to 75 mm (from the lower edge), followed by drying overnight (minimum 12 h) at 105 °C.

Post-chromatographic derivatization

1. Enzymatic digest: Still warm plates are sprayed with 1 mL of enzyme solution (250-fold diluted Quantum® Blueliquid 5G in 50 mM NaOAc pH 4.5) using the Derivatizer (pre-cleaned with water). After spraying, the plate is pre-incubated at ambient temperature for 5 min and then transferred to a TLC Plate Heater at 55 °C for 15 min.

2. Molybdate reagent: Plates are sprayed with 0.5 mL of molybdate reagent (5 mL of a 10 g/L ammonium molybdate heptahydrate aq. solution mixed with 200 µL of concentrated sulfuric acid freshly prepared every day) using the Derivatizer. Subsequently the plates are treated with UV light at 254 nm for 15 min.

Documentation

Images of the plate are captured with the TLC Visualizer in white light.

Densitometry

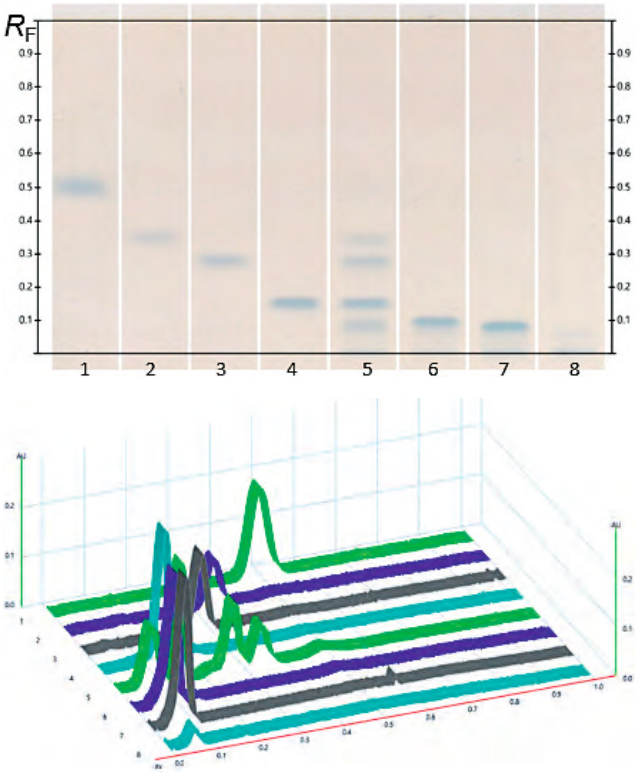
Absorbance measurement is performed with a DAD scanner [1] and with the TLC Scanner 4 at 774 nm, with a scanning speed of 5 mm/s, a data resolution of 25 µm/step, slit dimension 5.0 mm × 0.3 mm, spectra recording from 200 to 800 nm.

Results and discussion

This HPTLC method is suitable for the separation of $InsP_x$ pools as well as P_i. The isomers $Ins(3)P_1$, $Ins(2,4)P_2$, $Ins(1,4,5)P_3$ and free phosphate are baseline separated. $Ins(2,3,5,6)P_4$ and $Ins(1,3,4,5,6)P_5$ may be quantified by the peak splitting method. $InsP_6$ (track 8) shows two bands in a concentration-dependent manner. Presumably, the part that is present as an undissolved salt remains on the application line, while the free base migrates to an R_F value of 0.06 and thus co-migrates with $Ins(1,3,4,5,6)P_5$ (R_F = 0.07).

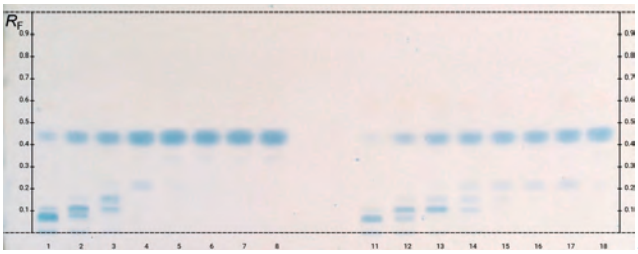
Acidic conditions or salt containing samples may affect R_F values, however not the overall separation of inositol phosphates. The quantification of the $InsP_x$ isomers can be performed by external standards and linear regression. For free phosphate, two linear ranges were found between 5–15 ng and 20–150 ng with correlation coefficients of 0.99 ([1] by using the Kubelka-Munk equation). Free phosphate was detected with a LOD and LOQ of 5.7 and 6.9 ng respectively.

The method is utilized to study the $InsP_x$ fingerprint of a phytase to evaluate its ability of phytic acid degradation. Our results show that the HPTLC is suitable for a rapid screening of inositol phosphates with a semi-high sample throughput. Accumulation of isomers can be detected as well as a quantitative phosphate release. The presented method is a useful tool for a fast, visual evaluation of novel phytases.



1

HPTLC chromatograms in white light (top) and densitograms at 774 nm (bottom) after derivatization; Track 1: P_i (100 ng), track 2: $Ins(3)P_1$ (500 ng), track 3: $Ins(2,4)P_2$, (300 ng) track 4: $Ins(1,4,5)P_3$ (300 ng), track 5: $InsP_1$-P_5, track 6: $Ins(2,3,5,6)P_4$ (300 ng), track 7: $Ins(1,3,4,5,6)P_5$ (300 ng), track 8: $Ins(1,2,3,4,5,6)P_6$ (100 ng).



2

*HPTLC fingerprints of InsPx (10 U*L^{-1}, 37 °C) of the phytase Quantum® Blue at pH 3.6 (tracks 1–8) and pH 5.5 (tracks 11–18) at the time points 5, 30, 60, 120, 180, 240, 300 min and 24 h in ascending order; Image from [1] (https://creativecommons.org/licenses/by/4.0/legalcode).*

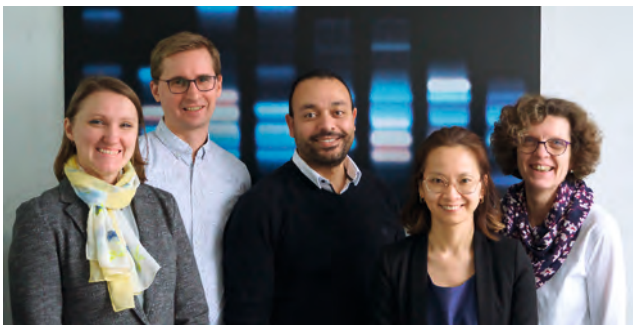
Editor´s note: The R_F values vary between the shown chromatograms. The presence of salts and acids, as well as the relative humidity influences the position of the separated $InsP_x$.

[1] C. Henninger *et al.*, *J Sci Food Agric.* (2023), https://doi.org/10.1002/jsf2.109.

Further information is available on request from the author.

Contact: Corinna Henninger, Offenburg University of Applied Sciences, Badstrasse 24, 77652 Offenburg, Germany, corinna.henninger@hs-offenburg.de

Detection of paraffin oil in milk using HPTLC PRO



From left: Dr. Kateryna Khokhlova, Dr. Stefan Weiss, Dr. Ehab Mahran, Dr. Tiên Do, Ilona Trettin

Introduction

Contamination of food with mineral oil products is of significant concern to food safety. Mineral oils can enter food either intentionally or unintentionally as contaminants. Mineral oils, known as MOH (mineral oil hydrocarbons) are intricate mixtures of hydrocarbons obtained from crude oil. They are divided into two fractions: 1. mineral oil aromatic hydrocarbons (MOAH), and 2. paraffin oil, also known as mineral oil saturated hydrocarbons (MOSH). MOSH consist of straight and branched open-chain alkanes (paraffins) and alkylated cycloalkanes (naphthenes). The diverse nature of these compounds presents a substantial challenge for analytical methods. For companies involved in milk processing, particular attention is given to detecting milk batches that might be contaminated with paraffin oil.

The screening method employed for this purpose needs to be as straightforward and rapid as possible. HPTLC, due to its ability to simultaneously separate multiple samples, is a suitable technique to fulfill these requirements.

The procedure described here involves isolating non-polar components from milk through liquid-liquid extraction. Following the approach of Wagner and Oellig [1], the MOSH fraction is subsequently separated and identified on the HPTLC plate using primuline as derivatization step. This method can detect 5.0 µg/mL of paraffin oil in milk.

Standard solution

To avoid the possibility of contamination from leaching processes, only glass laboratory equipment is utilized for preparation of standards and samples. During method development, technical grade paraffin oil was diluted with toluene to a concentration of 10.0 mg/mL.

Sample preparation

For calibration and repeatability, 2.0 mL of each milk sample containing 3.9 % milk fat is pipetted into glass centrifuge tubes using a volumetric glass pipette. These samples are spiked with the 10.0 mg/mL standard solution using a 10.0 µL glass syringe.

The sample is acidified with 0.4 mL of formic acid (≥98 %). After adding 3.0 mL of tert-butyl methyl ether and vortexing for 30 s, the sample is centrifuged for 5 min with 2790 x g. The organic supernatant is transferred into a glass vial and used as test solution.

Chromatogram layer

HPTLC plates silica gel 60 F_{254} (Supelco), 20 x 10 cm are used after pre-washing with cyclohexane up to 50 mm and drying for 30 min at 100 °C.

Impregnation

Prior to sample application, HPTLC plates are impregnated with primuline solution (75 mg/L in methanol) using the Chromatogram Immersion Device III (time 20 s, speed 1), and dried using the TLC Plate Heater at 100 °C for 30 min.

Sample application

6.0 µL of sample and standard solutions are applied as bands with the HPTLC PRO Module APPLICATION, band length 6.0 mm, distance from the left edge 18.0 mm, track distance 8.5 mm, distance from the lower edge 8.0 mm. The first rinsing step (solvent bottle 1) is performed with methanol – acetonitrile – isopropanol – water – formic acid 250:250:250:250:1 (*V/V*) and the second rinsing step (solvent bottle 2) with methanol – water 7:3 (*V/V*).

Chromatography

In the HPTLC PRO Module DEVELOPMENT, prior to the development the plates are pre-dried for 30 s, activated at 0–5 % relative humidity for 10 minutes

using a molecular sieve, and pre-conditioned with cyclohexane at a pump power of 35 % for 300 s. No conditioning step is used. Development with cyclohexane to the migration distance of 30 mm from the lower edge of the plate, followed by drying for 5 min.

Documentation

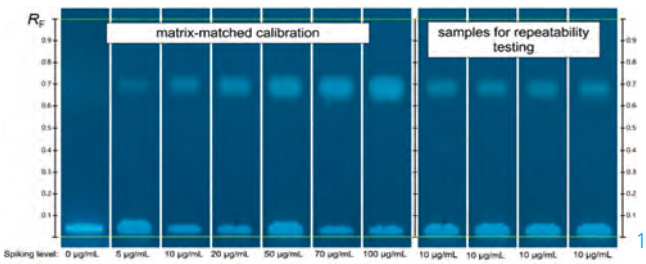
Images of the plates are captured with the TLC Visualizer 2 in UV 366 nm.

Results and discussion

A calibration curve ranging from 5.0 µg/mL to 100.0 µg/mL was created by spiking aliquots of a milk sample with paraffin oil. For this purpose, the 2.0 mL samples were spiked with 1.0 – 20.0 µL of the 10.0 mg/mL standard solution.

Due to the short development distance of 30 mm, the development time is only 2 min. Considering activation, pre-conditioning, and drying, the complete development cycle is 30 min. This means that if 16 samples are applied to one HPTLC plate, the separation time per sample is only 1.8 min (6.7 min including application). As the HPTLC PRO system autonomously moves the plate from one module to the next, there is no time wasted due to manual transfer between the individual HPTLC steps.

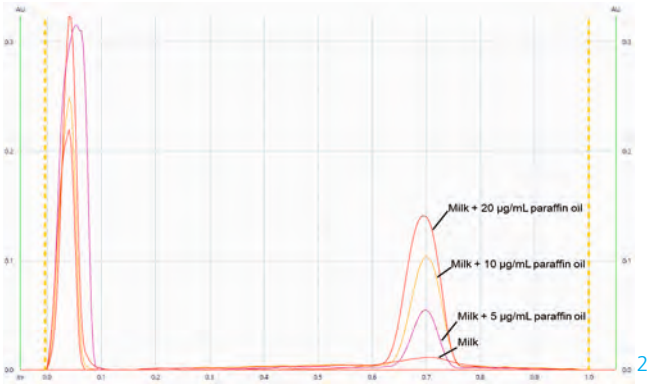
In the image of the developed plate in UV 366 nm, the first track is the not spiked milk sample, followed by the reference samples for the matrix-matched calibration and, on the last four tracks, the repeatability samples. The clear separation of the paraffin oil fraction from the other extracted fluorescent components is readily apparent. The lowest spike at 5.0 µg/mL is also clearly visible (second track from the left).



1

HPTLC chromatograms of matrix-matched calibration standards (from left: track 1–7) and repeatability samples (from left: track 8–11) in UV 366 nm after separation on the primuline impregnated HPTLC plate with a migration distance of 30 mm.

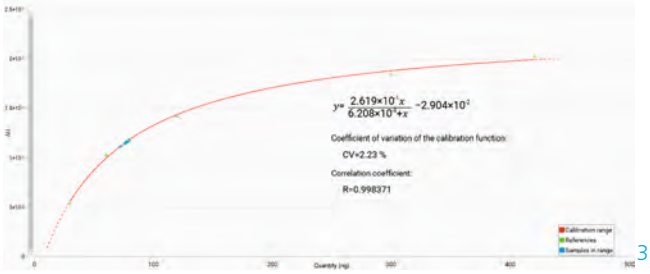
Using *visionCATS*, peak profiles from the image in UV 366 nm were generated for each individual track. Subsequently, the evaluation was conducted based on peak height.



2

Peak profiles from the image in UV 366 nm for the unspiked sample and the samples spiked with 5.0, 10.0 and 20.0 µg/mL of paraffin oil.

The calibration curve was generated using a Mime-2 function. The calibration from 5.0 µg/mL to 70.0 µg/mL includes the results obtained for the four spiked samples (blue cross).



3

Calibration curve (from 5.0 µg/mL to 70.0 µg/mL) from the peak heights using the Mime-2 function.

For the reproducibility of the extraction, an average value of 12.70 µg/mL, and a standard deviation (STDEV) of 0.51 µg/mL were obtained.

Results of the reproducibility and recovery tests

Samples	Concentration in µg/mL
1	12.90
2	12.01
3	12.69
4	13.20
Mean	12.70
STDEV	0.51

This study demonstrates the rapid and simple, yet sensitive detection of paraffin oil in milk.

[Note]: for a proper quantification, a linear calibration curve would be needed.

Further information upon request from the authors.

[1] M. Wagner and C. Oellig, J. Chromatogr. A ,1588 (2019) 48–57, https://doi.org/10.1016/j.chroma.2018.12.043.

Contact: Dr. Tiên Do, CAMAG, Sonnenmattstrasse 11, 4132 Muttenz, Switzerland, lab@camag.com

HPTLC fingerprinting for quality assessment of Ashwagandha formulations

Mr. Aniket Jadhav, an application chemist working under the guidance of Mr. Akshay Charegaonkar, Managing Director, is employed at Anchrom Enterprises Pvt. Ltd. in Mumbai, India. The company specializes in instrumental planar chromatography and is dedicated to the development of innovative quantitative and regulatory-compliant analytical methods for various sectors including pharmaceutical formulations, APIs, herbal products, food products, organic intermediates, dyes, and more.

Mr. Jadhav utilizes the advantages of HPTLC due to its attributes as a rapid, easy, cost-effective, and versatile "visible chromatography" technique. HPTLC is distinguished by its risk-free nature, allowing for multiple detections without the need for repeated chromatography.

Introduction

Withania somnifera, commonly known as Ashwagandha, is a versatile herb deeply rooted in traditional and modern herbal medicine. This herb, belonging to the Solanaceae family, holds a significant place in the cultures of India and beyond. Ashwagandha is recognized for its potential therapeutic properties, including adaptogenic, anti-inflammatory, antioxidant, and stress-reducing effects. Moreover, it has gained prominence in the pharmaceutical and dietary supplement industries due to its various health benefits. The quality and authenticity of Ashwagandha formulations can be influenced by several factors, including the source of cultivation, processing methods, and storage conditions. This valuable herb is, however, vulnerable to potential adulteration and contamination, which can compromise its efficacy and safety. To ensure the purity and integrity of Ashwagandha formulations, it is essential to develop analytical methods capable of detecting adulteration in these products. HPTLC has emerged as a valuable tool in this regard, offering a cost-effective and efficient means of separating and analyzing complex mixtures of chemical components.

HPTLC offers a rapid, cost-effective, and efficient method for distinguishing pure Ashwagandha BRM from potential adulterants in Ashwagandha products available in the market and contributing to the overall integrity and reliability of herbal products in the industry. By analyzing multiple samples simultaneously on a single plate, this technique saves time and resources while minimizing solvent consumption and waste production. It serves as a vital quality control measure, ensuring the authenticity and purity of Ashwagandha formulations, thereby enhances consumer trust and upholds the herbal industry's reputation.

Standard solution

1.0 g of *Withania somnifera* root (BRM) is dissolved in 10 mL methanol.

Sample preparation

1.0 g of *Withania somnifera* marketed formulations are dissolved in 10 mL of methanol. The samples were vortexed thoroughly and centrifuged at 2000 rpm for 5 min. After centrifugation, the supernatant is collected and used for the application.

Chromatogram layer

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

Sample application

2.0 and 10.0 µL of sample solutions and 10.0 µL of standard solutions are applied as bands with the Automatic TLC Sampler (ATS 4), 13 tracks, band length 8.0 mm, distance from the left edge 15.0 mm, track distance 13.4 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 solution of magnesium chloride, development with toluene – ethyl acetate – glacial acetic acid 55:45:3 (*V/V*) to the migration distance of 70 mm (from the lower edge), followed by drying for 5 min.

Post-chromatographic derivatization

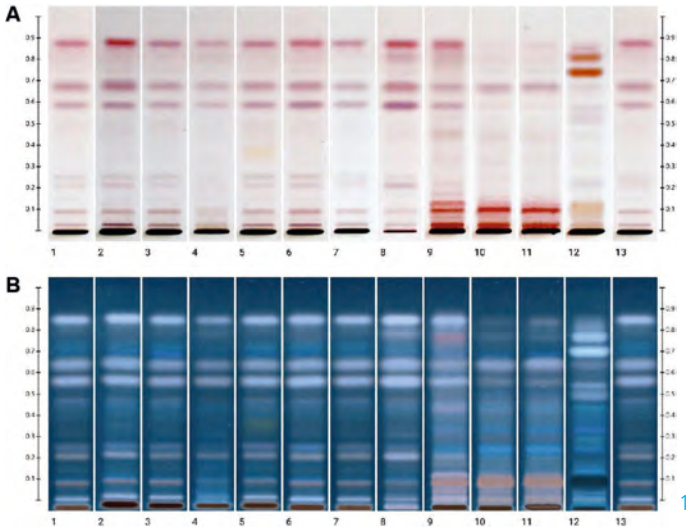
The plate is sprayed with 3 mL of sulfuric acid reagent (180 mL ice-cold methanol are mixed with 20 mL of concentrated sulfuric acid and allowed to cool to room temperature) using the Derivatizer. After spraying, the plate is heated at 110 °C for 3 min using the TLC Plate Heater.

Documentation

Images of the plate are captured with the TLC Visualizer in UV 366 nm and white light after derivatization.

Results and discussion

In the comparison of *Withania somnifera* root (BRM) with various marketed samples of *Withania somnifera* (Ashwagandha), several significant observations were made. Firstly, the visible images revealed that the BRM standard exhibited seven intense bands, setting it apart from the other samples. Notably, marketed samples 1, 6, 7, 8, 9, and 10 displayed a similar pattern to the BRM standard, indicating a degree of similarity in their composition. However, marketed samples 9 and 7 exhibited some extra bands at R_F 0.37 and R_F 0.81, respectively, suggesting potential variations in their chemical profiles. Furthermore, marketed sample 2 displayed two additional bands at R_F 0.13 and 0.46, which distinguished it from the others. On the other hand, marketed samples 3 and 4 shared similarity with each other but did not match with the fingerprinting pattern of the BRM. Lastly, marketed sample 5 exhibited a distinct profile compared to the botanical standard, featuring extra bands at R_F 0.53, 0.74, and 0.82. These findings suggest variations in chemical composition among the analyzed Ashwagandha samples, emphasizing the importance of precise quality control in herbal product assessment and standardization.



1

HPTLC fingerprints: in white light (A) and UV 366 nm (B) after derivatization with sulfuric acid reagent; Tracks 1, 7 & 13: Botanical reference material; track 2: sample 1, track 3: sample 6, track 4: sample 8, track 5: sample 9, track 6: sample 10, track 8: sample 7, track 9: sample 2, track 10: sample 3, track 11: sample 4, track 12: sample 5

[1] HPTLC Association - *Withania somnifera* root

Further information is available on request from the author.

Contact: Mr. Akshay Charegaonkar,
A-101, Shree Aniket Apartments, Navghar Road, Mulund East,
Mumbai, Maharashtra 400081, India,
hptlc@anchrom.in, www.anchrom.in

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