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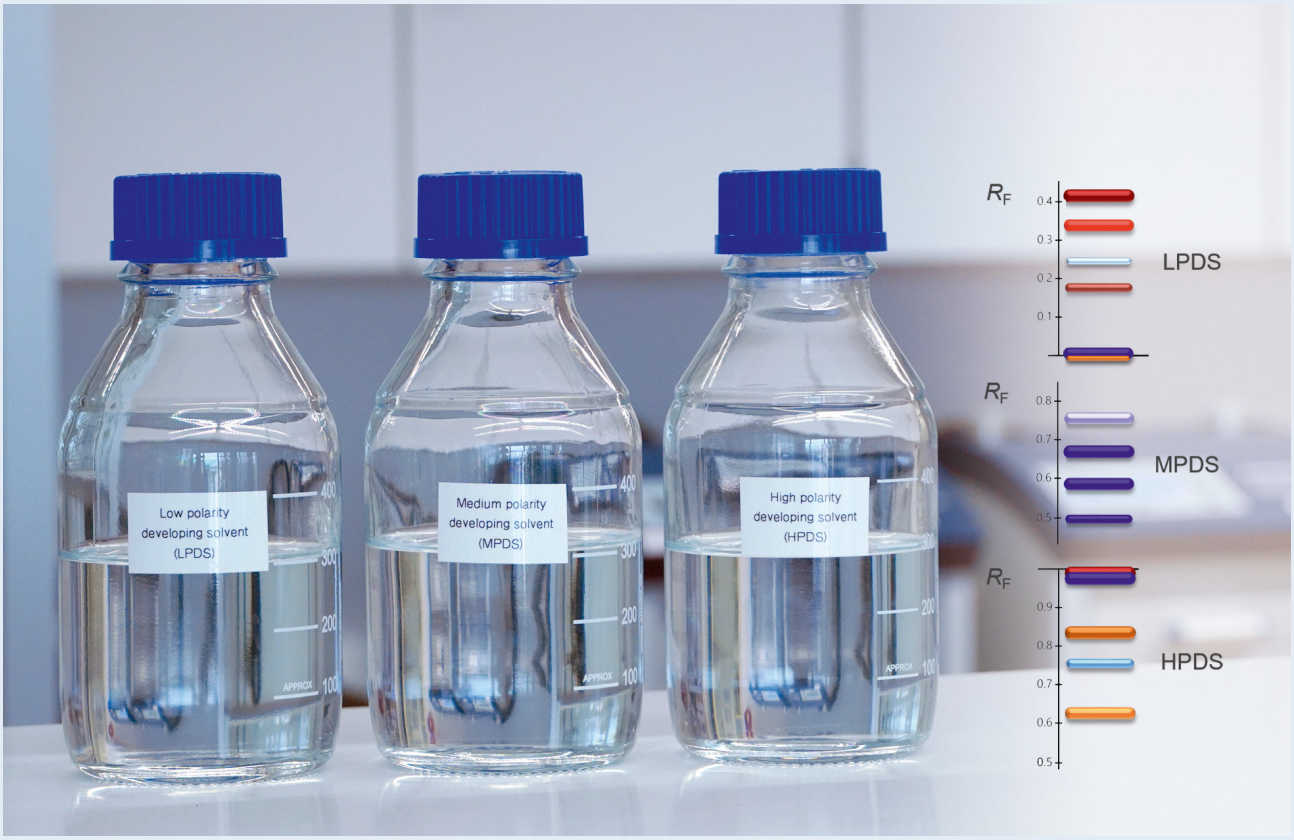

Complementary Developing Solvents (CDS): A new concept for routine analysis

Featured applications from France in this anniversary edition:

- Quantification on plate and coupling with preparative LC
- Development and validation of an HPTLC-DPPH assay method for the acteoside content of Ribwort ipowder®
- Characterization of enzymes from plant lipid metabolism

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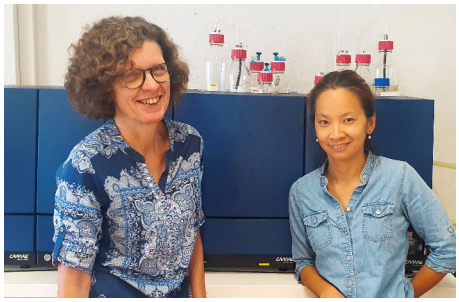
HPTLC routine analysis using complementary developing solvents

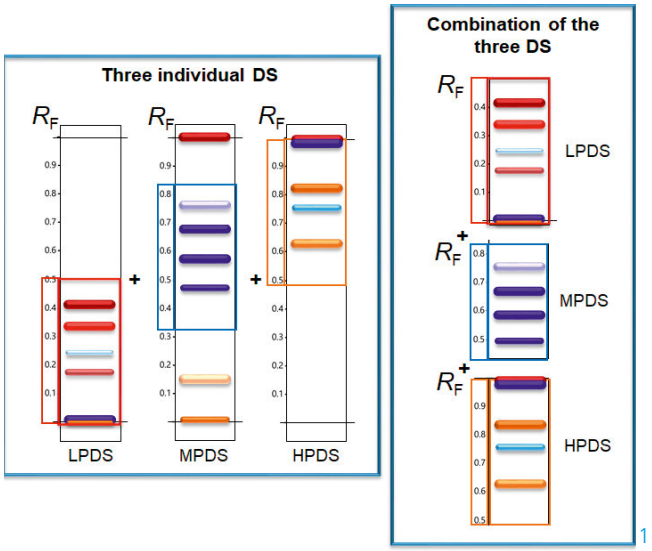


Ilona Trettin and Dr. Tiên Do

Introduction

In quality control with HPTLC, a specific method using optimized developing solvents is generally used for each kind of sample. In order to simplify routine analysis, the lab team at CAMAG has developed the complementary developing solvents (CDS) concept based on one solvent of low polarity (LPDS), one of medium polarity (MPDS), and one of high polarity (HPDS). With these three developing solvents (DS), each on a separate plate and targeting compounds of different polarity, the same complex sample could be spread over up to three times the separation distance on a single plate, making available more information about the sample's composition. Single substances can be characterized with three R_F values instead of one. Even though this approach triples the analytical workload (3 analyses instead of one), it may be considered, that routine work with multiple and diverse samples can be simplified and maintenance of methods, plates, solvents and standards can be kept to a minimum, particularly if the process is automated. Identification of individual compounds will be more certain. A further advantage of the concept is that all samples can be compared with any other sample that has been previously analyzed with the same CDS and data could be compiled in a database for treatment with advanced algorithms.

In their paper [1] the researchers at CAMAG describe the development, validation and application of a CDS, applicable to a large number of very diverse samples including individual compounds and complex herbal materials. In combination with thorough standardization, the concept could help positioning HPTLC as a very powerful, general, and medium to high throughput technique for routine analysis and sophisticated research.



Visualization of the CDS and its fingerprints

Standard solutions

The Universal HPTLC mix (UHM) was prepared in house according to [2]. With the UHM, HPTLC laboratories have a single solution, applicable as system suitability test to a wide range of chromatographic systems.

Sample preparation

Powdered herbal drugs and finished products were prepared in methanol using 10 min sonication followed by 5 min centrifugation. *Ginkgo biloba*, *Camellia sinensis*, *Styphnolobium japonica* and *Piper nigrum* were prepared at 100 mg/mL, *Curcuma longa* at 66.7 mg/mL, Angelica samples at 200 mg/mL, and the poly-herbal formulation at 50 mg/mL.

Chromatogram layer

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

Sample application

Samples are applied as bands with the Automatic TLC Sampler (ATS 4), 15 tracks, band length 8.0 mm, distance from left edge 20.0 mm, distance from lower edge 8.0 mm.

Chromatography

Plates were developed with the three developing solvents in the ADC 2 with activation of the plate at 33% relative humidity for 10 min using a saturated solution of magnesium chloride. LPDS was used without saturation, whereas MPDS and HPDS were used with 20 min chamber saturation (with filter paper). The developing distance for all three methods was 70 mm (from the lower edge). Plates were dried for 5 min.

Post-chromatographic derivatization

Natural product (NP) reagent (1.0 g of 2-aminoethyl diphenylborinate in 100.0 mL of methanol) is used as derivatization reagent for the identification of *Camelia sinensis*, *Styphnolobium japonicum* and *Ginkgo biloba*. For *Ginkgo biloba*, the derivatization with NP is followed by anisaldehyde sulfuric acid (AS) reagent [slowly and carefully 170.0 mL of ice-cooled methanol are mixed with 20.0 mL of acetic acid and 10.0 mL of sulfuric acid; mixture is allowed to cool to room temperature, then 1.0 mL of anisaldehyde (*p*-methoxybenzaldehyde) is added]. Only AS reagent is used for the identification of *Curcuma longa* and *Piper nigrum*.

Documentation

Images are captured with the TLC Visualizer 2 in UV 254 nm, UV 366 nm, and white light prior to derivatization, and UV 366 nm, and white light after derivatization (when needed).

Densitometry

For the system suitability test using the UHM, TLC Scanner 4 and *visionCATS* are used in absorbance mode at 254 nm and in fluorescence mode at 366 >/400 nm, with slit dimension 5.00 x 0.20 mm and scanning speed of 50 mm/s.

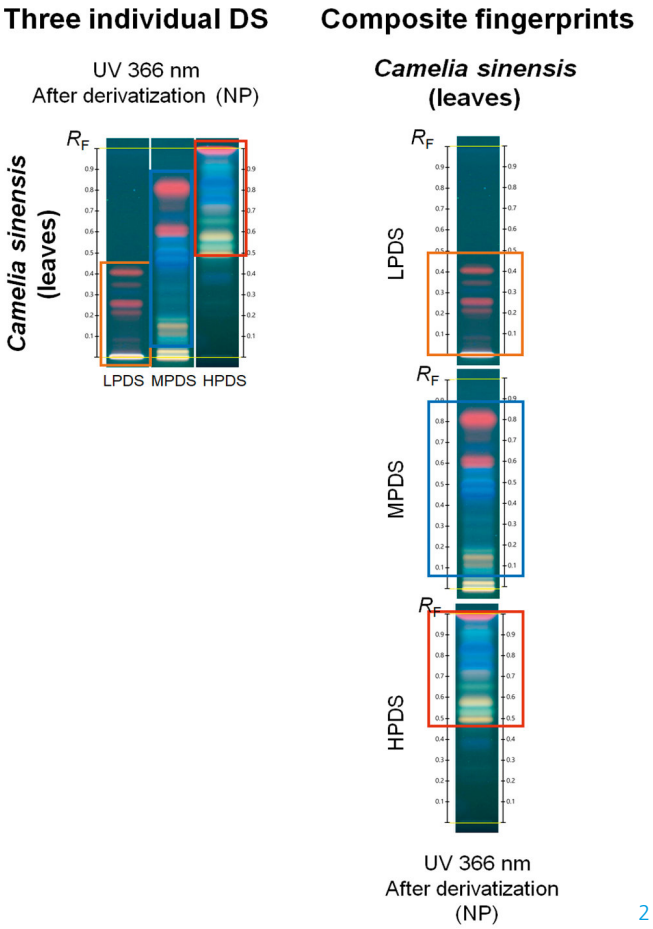
Results and discussion

The solvents selected for the CDS had to meet the following criteria: be of minimal hazard, stable, and easily available, to cover all selectivity groups according to Snyder, and include a broad range of polarity. The composition and properties of the CDS are shown in the table.

Composition and properties of the CDSs

	Description	Selectivity groups	P'	Dev. time (min)
LPDS	toluene, ethyl acetate 9:1 (*V/V*); activation 33% r.H. (10 min); no saturation	VII, VIa	2.60	17
MPDS	cyclopentyl methyl ether, tetrahydrofuran, water, formic acid 40:24:1:1 (*V/V*); activation 33% r.H. (10 min); saturation (20 min, saturation pad)	I, III, VIII, IV	3.30	12
HPDS	ethanol, dichloromethane, water, formic acid 16:16:4:1 (*V/V*); activation 33% r.H. (10 min); saturation (20 min, saturation pad)	II, V, VIII, IV	4.96	30
P': polarity index				

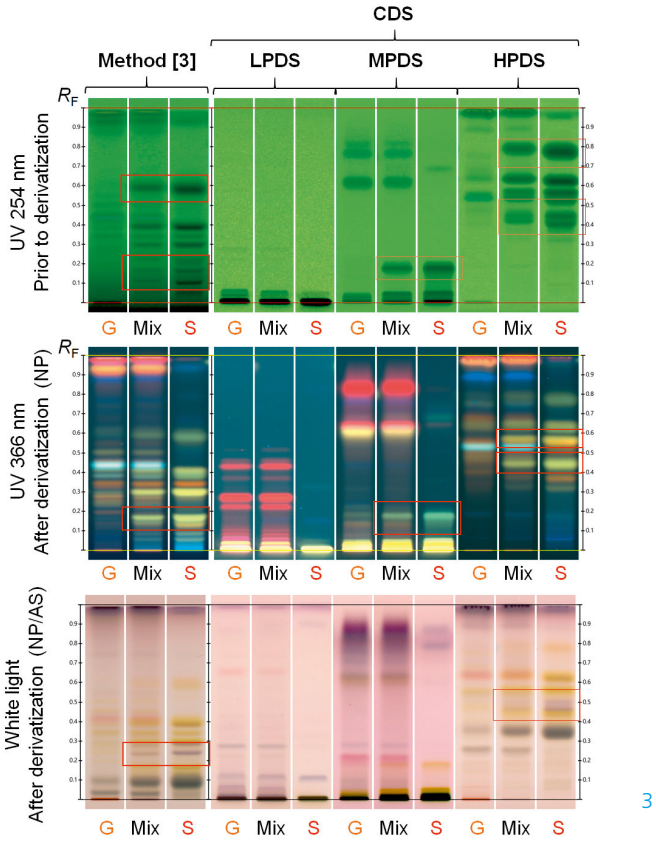
The power of the CDS concept is illustrated with HPTLC fingerprints for identification of herbal drugs, herbal products, and poly-herbal formulations. Green tea leaves, for example, produce a different fingerprint with each of the DS zooming into specific polarities of the sample composition. The composite fingerprint gives complementary information emulating an extended developing distance.



2

HPTLC fingerprints of green tea leaves obtained with the CDS

HPTLC can easily detect adulteration of one herbal drug with another, using optimized developing solvents. For example, a method from the HPTLC Association [3] detects adulteration of *Ginkgo biloba* leaves with fruits of *Styphnolobium japonicum*. The CDS achieves the same goal, but offers even more certainty based on the data obtained with MPDS and HPDS.



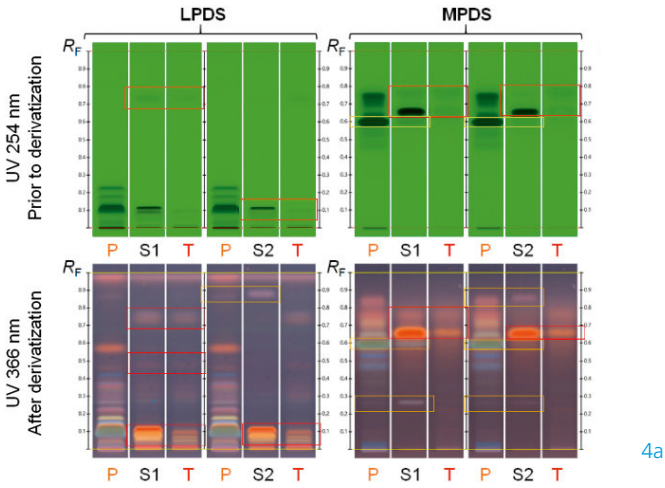
3

G: *Ginkgo biloba* leaves
Mix: *Ginkgo biloba* leaves 80%; *Styphnolobium japonicum* fruits 20%
S: *Styphnolobium japonicum* fruits

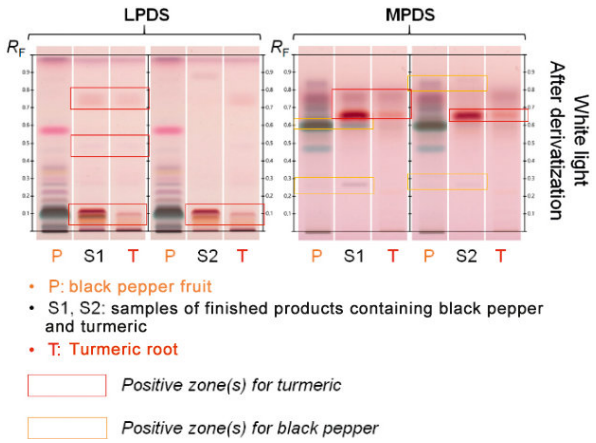
Positive zone(s) for Styphnolobium japonicum

Detection of 20% Styphnolobium japonicum fruit in Ginkgo biloba leaves with method [3] and CDS

Poly-herbal formulations such as products containing *Curcuma longa* and *Piper nigrum* are generally identified based on specific markers. The CDS can identify with certainty the presence of curcuminoids for turmeric and piperine for black pepper.



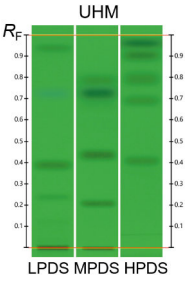
4a



4b

Fingerprints of Curcuma longa and Piper nigrum in comparison to those of two poly-herbal products

The CDS has been qualified using the universal HPTLC mix (UHM). A maximum margin of error of 0.014 was determined for the relevant zones.



Separation of the UHM components with the CDS

5

Conclusion

The examples above illustrate the potential of the CDS for replacing the established methods for identification of herbal materials. Additional information concerning the chromatographic behavior of representative substances from different chemical classes is presented in the original paper [1].

When combined with fully automated chromatography, the CDS concept may become the basis for new applications of HPTLC in routine analysis and sophisticated research.

[1] T.K.T. Do *et al.* (2022) JPC https://doi.org/10.1007/s00764-022-00185-1.

[2] T.K.T. Do *et al.* (2021) J. Chromatogr. A. 1638 https://doi.org/10.1016/j.chroma.2020.461830.

[3] HPTLC Association, Identification method of Ginkgo biloba, Leaf and leaf extract (flavonoids), (n.d.). https://www.hptlc-association.org/methods/methods_for_identification_of_herbals.cfm.

Further information is available on request from the authors.

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6

CAMAG® HPTLC PRO Module DERIVATIZATION

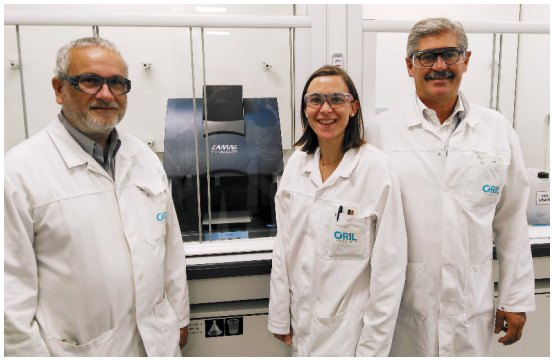
The HPTLC PRO Module DERIVATIZATION combines two steps in a single and fully automated device: high-precision spraying of derivatization reagents and heating of the plate. Employing the patented micro-droplet spraying technology at a slight underpressure enables maximum homogeneity in applying reagents at a very low consumption rate, while the integrated heating unit ensures a uniform heat distribution across the plate. To suit the viscosity of the spraying reagents, four different nozzles are available. Equipped with a fully automated nozzle changer and a cleaning station, the HPTLC PRO Module DERIVATIZATION effectively avoids cross-contamination.

Key features:

- Part of the fully automated HPTLC PRO System
- Nozzle changer to choose nozzles from three different nozzle parking positions
- Cleaning station
- Integrated plate heating unit
- Handling of two different reagents
- HPTLC glass plates (20 x 10 cm)
- Software-controlled by *visionCATS*

Further information:
www.camag.com/PROderivatization

Use of TLC, HPTLC, and HPTLC-MS during production and purification processes of active ingredients and their impurities



Didier Rigollet, Amélie Havard and Daniel Dron

Didier Rigollet, Amélie Havard and Daniel Dron are working at the R&D department Analytical Innovative Technologies of the Industrial Research Centre at Oril Industrie, in Bolbec, France (Servier group). The use of HPTLC in Oril started even earlier than the foundation of Chromacim, including trainings at CAMAG organised by Pierre Bernard-Savary, where Daniel Dron participated already more than 20 years ago. The R&D team is specialized in purification processes of intermediates and active pharmaceutical ingredients (APIs) for toxicological, galenical or clinical studies. Part of their work is devoted to the isolation of impurities and production of APIs or impurity reference batches. In 2018, the team launched their preparative chromatography service InnoPrep™, dedicated to small and large-scale purifications. Intermediates, APIs and impurities are characterized by MS and NMR. Quantitative analysis by NMR is also performed on these molecules.

Introduction

Servier, an independent international pharmaceutical group, is committed to therapeutic progress for the benefit of patients.

Their goal is to speed up the development of new molecules in order to bring a new molecular entity to market every 3 years, particularly in the field of oncology. Preparative chromatography is, therefore, a method of choice in R&D to provide pure products for the first pharmacological, toxicological and clinical studies in a very short time. This technique also makes it possible to isolate impurities present at low levels in active ingredients whose complex structure does not allow rapid synthesis and to provide a batch within the time limits set for toxicological studies.

About 75% of the purifications at Oril Industrie are done with silica gel. The necessary conditions for an efficient purification are determined using TLC. Then, the purification progress by preparative column chromatography is checked by HPTLC. Twenty fractions can be analyzed within one hour. TLC/HPTLC is the method of choice due to its simplicity, rapidness and the successful scale up from TLC to preparative separations. The HPTLC-MS carried out beforehand allows targeting the molecule sought in often very complex mixtures.

Sample preparation

Crude product (0.05 g) is dissolved in 5.0 mL of ethyl acetate.

Chromatogram layer

For method development (optimization of purification conditions) TLC plates silica gel 60 F_{254} (Merck), 20 × 5 cm are used, while quantification and coupling to mass spectrometry is done on HPTLC plates silica gel 60 F_{254} s (Merck), 20 × 10 cm.

Sample application

Samples are applied as bands with the Automatic TLC Sampler (ATS 4), two tracks for TLC and up to 20 tracks for HPTLC, band length 8.0 mm, sample volumes of 1.0–15.0 µL.

Chromatography

Plates are developed in the Twin Trough Chamber 20 × 20 cm (TLC) or 20 × 10 cm (HPTLC) with chamber saturation (with filter paper) for 20 min with different developing solvents to the separation distance of 100 mm (from the lower edge) for TLC and 50 mm for HPTLC, followed by drying in a stream of cold air for 5 min.

Documentation

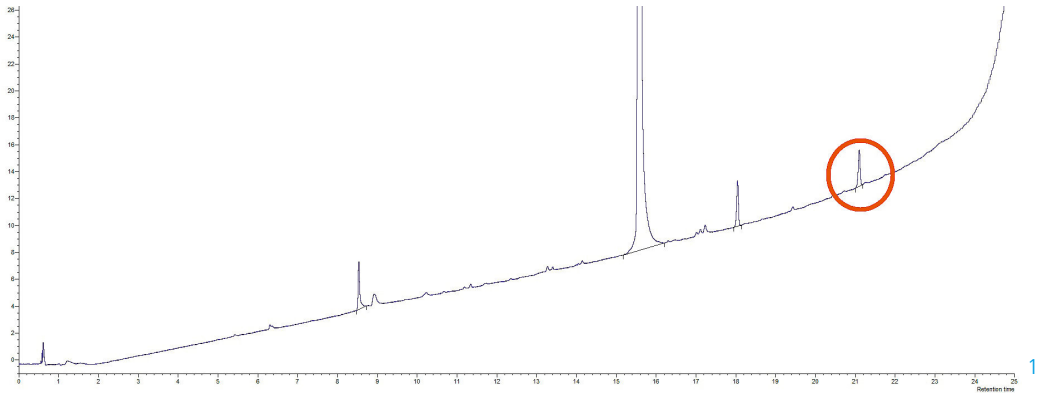
Images of the plates are captured with the TLC Visualizer in UV 254 nm and white light.

Mass spectrometry

Zones are eluted with the TLC-MS Interface (oval elution head) at a flow rate of 0.2 mL/min with methanol – water 1:1 (*V/V*) into a Q-TOF-MS (Xevo® G2-S QTof, Waters), operating in positive ionization mode (*m/z* 50–1200).

Results and discussion

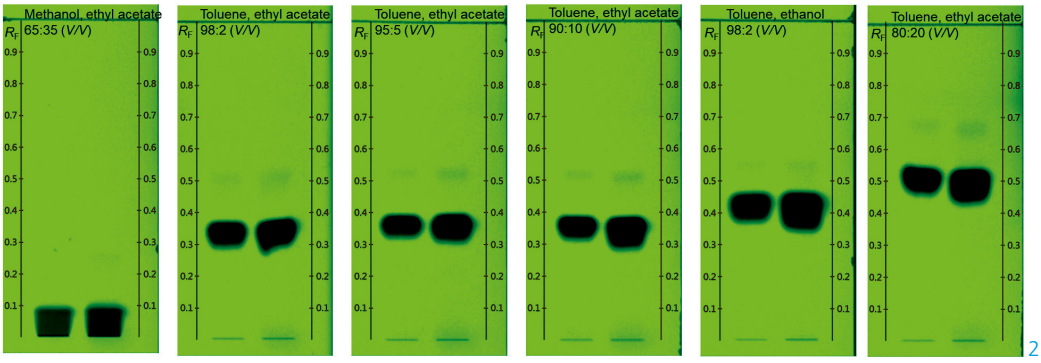
The objective of this study was to isolate a sufficient quantity of an impurity present at a content of 0.35% in a batch of an intermediate in a product under development. The aim was to confirm its structure and to carry out toxicological tests. The nature of this impurity was determined beforehand by LC-MS. Its structure being complex, a synthesis would be too time-consuming. The conditions used in RP-HPLC are too complex for direct transfer to preparative chromatography (expensive stationary phase). The mobile phase used was water + 0.1% methane sulfonic acid and acetonitrile + 0.1% methane sulfonic acid is too complexed for the isolation after preparative chromatography.



1

RP-LC-UV Chromatogram of the crude product

TLC was selected for method development to separate the major impurity from the other compounds with a reasonable R_F value allowing an efficient purification.



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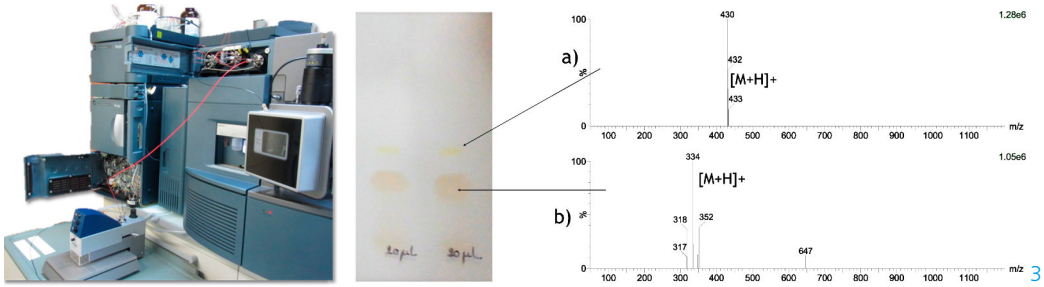
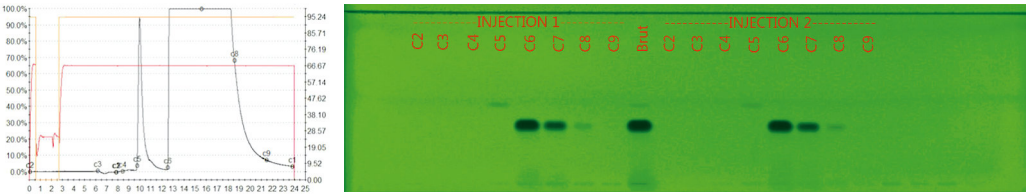
TLC chromatograms of the crude product in UV 254 nm obtained with different developing solvents

Mass spectra were recorded to characterize the different compounds (main substance at R_F 0.25 and impurity at R_F 0.38).



3 *Instruments used for TLC-MS (left); TLC chromatogram of the main substance and the impurity in white light (middle); mass spectra of the selected zones (a: impurity, b: main substance) measured in positive ionization mode (right)*

The purification of the crude product (two injections of 90 g dissolved in toluene) on a 20-cm column (packed at 40 bars with 6 kg silica gel 60, 15–40 µm, Merck) at a flow rate of 2.0 L/min with toluene – ethyl acetate 95:5 (*V/V*), was monitored online with UV 290 nm detection and in parallel offline by HPTLC.



4 *Online monitoring of the purification process by LC-UV (290 nm, left) versus offline by HPTLC-UV (HPTLC chromatogram of the individual fractions at 254 nm, right; C2-C9 are fractions collected during purification and C5 corresponds to the impurity)*

The different fractions of the target impurity were collected, and the combined fractions were analyzed by NMR. 670 mg of impurity was obtained (yield: 0.35%) purity > 99%. The quantity obtained after purification on column was also consistent with the estimated content in analytical HPLC.

For Oril, the use of HPTLC has a big positive impact on the production costs, with a benefit of thousands of Euros per year. This is due to the use of HPTLC for various optimizations of the manufacturing process and of raw materials external supply.

Further information is available on request from the authors.

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20 years Chromacim

Pierre Bernard-Savary and Jérémy Mercier in Chromacim's application lab

Chromacim, CAMAG's distributor for France looks back on a 20 years history. In March 2002, Chromacim was founded with the aim of representing exclusively CAMAG's instruments there. It's founder Pierre Bernard-Savary has not only taken care of sales and service, but has also been very committed to exchanges among users and to develop HPTLC at its most advanced level.

As a counterweight to the UPLC-MS mainstream in the field of separation sciences, Chromacim's activities are dedicated to HPTLC, and more precisely to CAMAG equipment. Pierre started his career in the mid-1980s at Merck France, in partnership with Bruker, for the low pressure gradient analytical-preparative HPLC. After two years as sales manager, he was entrusted with the TLC activity as product manager. At the end of 1998, he started an independent activity of training dedicated to HPTLC and founded the Club de CCM (Club de Chromatographie sur Couche Mince) together with a small group of friends, of which he is still the president. Based on this independent French non-profit association he also organizes and chairs the series of so far eight International Symposia on HPTLC, from 2003 in Lyon until 2018 in Bangkok. At the end of 2001, he left the American group VWR, to which Merck sold its chromatography activities, and which he felt was too far away from the historical Merck he joined initially. At the beginning of 2002, CAMAG proposed to him to found a company dedicated to the promotion of its instruments and their technical support for France. Thus Chromacim SAS was born.

Why "Chromacim"? "The name "Chromacim" refers first of all to "chromatography", and CiM hints at the top of the mountains – "cime" in French – here in Chartreuse. The official typography of "Chroma-CiM" also highlights three letters – the C, C, and M – recalling the acronym of the famous method. For two years, Pierre worked alone, with the support of CAMAG, for the administrative management of the company. At the end of 2004, he recruited a service engineer, followed in 2006 with a first commercial engineer. Three years later, Chromacim set up its offices and warehouse in the heart of the high-tech industrial zone Centr'Alp, in Moirans, with the company's headquarters remaining in the Chartreuse natural park.

Pierre Bernard-Savary (right) during early days with his first two employees in their new office (2006)

Chromacim was able to grow in the first few years and soon had the first thousand instruments installed in France. The current team includes Jérémy Mercier who has moved within the company from technical sales to leading the sales activities. To better serve the customer needs, an application lab was established in 2018 in Grabels, close to Montpellier. Questions from customers can now be examined; instrument demonstrations and courses can be held there. Today Pierre and his team run numerous trainings in companies, schools and universities around the country. They also contribute yearly with posters and lectures to more than 10 scientific congresses, mainly in France.

We thank the colleagues at Chromacim for their commitment to represent CAMAG in France and their enthusiasm for HPTLC.

Dr. Markus Wyss
CEO CAMAG

Development and validation of an HPTLC-DPPH assay method for the acteoside content of Ribwort ipowder®

From left: C. Cotte, M. Aboussif, L. Berthomier, V. Bardot (from PiLeJe)

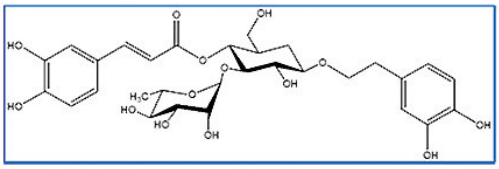
Authors note:
J.-M. Roussel (Consultant), M. Dubourdeaux (PiLeJe), S. Holowacz (PiLeJe), and P. Bernard-Savary (Chromacim) also contributed to this work.

The French company PiLeJe Industrie develops liquid and dry plant-derived ingredients using patented procedures and in-house developed processes. Their products are mainly used in the food supplements industry. The long-term collaboration with Chromacim led to the important development of HPTLC in their laboratory. This article addresses the question, whether it is possible to validate the activity assay of one major compound through its activity only, using the best international current standard.

Introduction

Ribwort plantain (*Plantago lanceolata* L.) is a common grassland plant traditionally used for its therapeutic properties. The leaves are used in many European countries for the symptomatic treatment of colds and inflammation of the mouth and throat. Biological activities of *P. lanceolata* include anti-histaminic, anti-spasmodic, anti-nociceptive, neuro-protective, metabolic, and gastro-protective activities. Acteoside is a phenyl-propanoid glycoside, well known for its antioxidant and anti-inflammatory properties, which is commonly used as a marker.

1



2

Ribwort ipowder® is a plant infusion concentrated on plant totum, a proprietary 100% plant-based product developed by PiLeJe Industrie, made from dried *P. lanceolata* according to a patented process [1, 2].

Quantification was already shown with standard detection and published in a previous issue of the CBS. Our goal was to prove that this type of activity detection is reliable and transferable to quality assurance with a proper validation package. This needs a level of knowledge that we have developed rather quickly with the help of our partners.

The objectives of this work were 1) to develop an HPTLC method using the 2,2-diphenyl 1-picryl-hydrazyle (DPPH*) effect-directed chemical reaction for the detection of the antioxidant activity of acteoside for quality control of industrial dry extracts of *P. lanceolata* and 2) to demonstrate the applicability of the concept of Life Cycle Management of analytical methods to quantitative HPTLC-DPPH* methods.

Standard solution

An acteoside standard is dissolved in methanol at a concentration of 17.40 µg/mL in methanol.

Sample preparation

500 mg of Ribwort ipowder® (PiLeJe Industrie) are extracted with 40 mL of ethanol – water 50:50

(*V/V*) by sonication at 60 °C for 10 min, then filtered and transferred to a 50 mL volumetric flask and filled up to the mark. The solution is diluted 5-fold for application.

Chromatogram layer

HPTLC plates silica gel 60, 20 x 10 cm are used.

Sample application

4.0 µL of sample and standard solutions are applied as bands with the Automatic TLC Sampler (ATS 4), 20 tracks, band length 8.0 mm, distance from left edge 22.0 mm, distance from lower edge 8.0 mm.

Chromatography

Plates are developed with ethyl acetate – water – acetic acid – formic acid 100:27:11:11 (*V/V*) to 70 mm (from the lower edge) in the ADC 2 with chamber saturation (20 min, with filter paper) and after activation at 33% relative humidity for 10 min using a saturated aqueous solution of magnesium chloride.

Post-chromatographic derivatization

After drying for 10 min, the plates are immersed into DPPH* reagent (0.5 mM methanolic solution of 2,2-diphenyl-1-picrylhydrazyl, immersion speed 5 cm/s, immersion time 5 s) with the Chromatogram Immersion Device 3. The plates are dried at room temperature in the dark for 90 s and then heated at 60 °C for 30 s (TLC Plate Heater 3).

Documentation

Images of the plates are captured with the TLC Visualizer in white light after derivatization.

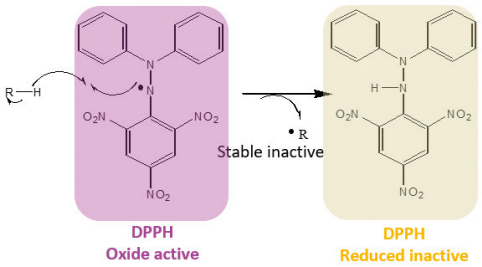
Densitometry

Fluorescence mode is used for measurement at 517 nm (tungsten lamp) with TLC Scanner 4 and *visionCATS* to obtain a positive response of the peaks of interest.

Results and discussion

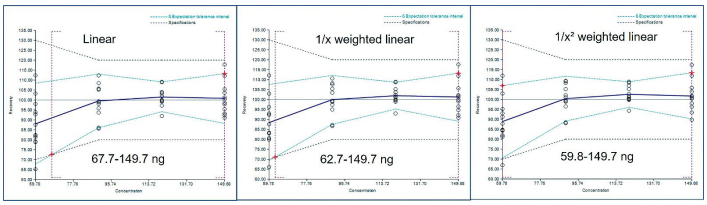
Analytical methods used for quality control of plants and plant extracts are usually based on the identification and quantification of chemical markers to manage batch reproducibility and efficacy. To measure the concentration of acteoside in Ribwort ipowder®, the HPTLC DDPH* assay was applied. The assay determines the free radical scavenging activity of the plant extract in solution.

Antioxidative properties - DPPH



3

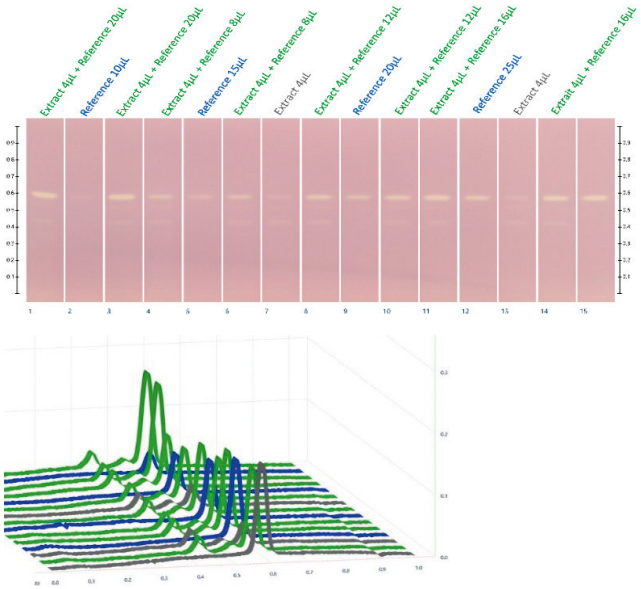
The first step of this work was the selection of the Analytical Target Profile (ATP) and the determination of the Target Measurement Uncertainty (TMU) taking into account the quality control requirements for such extracts and the applicable range of the detection method. Once the desired range was established, an evaluation of the calibration function was conducted using linear, 1/x and $1/x^2$ weighted linear calibration models and those three models were used to assess accuracy of the method (trueness and precision) by means of accuracy profiles [3]. The $1/x^2$ weighted linear calibration function showed the best performance in the tested range, both in terms of accuracy and uncertainty of measurement.



4

Accuracy profiles obtained with the selected calibration function, for each function the accuracy limits are given by the vertical doted lines.

The method requirement was to assay acteoside amounts around 1.0–2.0% (*W/W*) in industrial dry extracts of Ribwort plantain with an acceptance criterion of ±20.0% difference to the true value for the ATP (defined as the combination of the trueness (bias) and precision characteristics), and a TMU of less than 20.0–25.0% relative uncertainty, according to the quality control needs.



5

6

HPTLC chromatogram in white light and densitograms measured in fluorescence mode at 517 nm with a tungsten lamp after DPPH assay of standards and plantain leaf extract*

Due to the lack of reference samples, spiked samples were used to evaluate the accuracy of the method by means of Total Analytical Error (TAE) determination, using prediction intervals calculation for the selected calibration functions. For quality control, the calibration function with the best performance level in accordance with the product specifications was chosen by estimating the Measurement Uncertainty (MU).

As Life Cycle Management of the method also includes its routine use, the MU was checked on spiked and non-spiked extract samples at different dilution levels, in order to verify the accordance of results between those samples, and to prepare a replication strategy for the routine method. Statistical calculations were performed with NeoLiCy® software for analytical methods' life cycle statistical assessment (NeoLiCy, Marseille-Mâcon, France). The tested dilutions did not show any significant effect on the calculated spiked amount and any significant impact on the extract calculated concentrations. To take care of the measurement dispersion we included repeated sample preparation and measurement in the analytical procedure.

This work demonstrated that the concept of Life Cycle Management of analytical methods can successfully be applied to a HPTLC-DPPH* method, even in the case of complex matrices such as plant extracts, from the definition of the ATP and TMU to the reflection on the replication strategy to be applied in quality control.

The method developed for the quantification of acteoside in Ribwort plantain is applicable in a working range from 75.0 to 225.0 ng of acteoside and fit for purpose for use in quality control laboratories.

This study showed the suitability of HPTLC in this domain. Furthermore, the partnership with Chromacim and NéoLiCy showed its efficiency, within a cumulative working time of less than two weeks only, including statistics and matrix effect evaluation. This method is therefore ready to be transferred to the quality control laboratory of the PiLeJe Group.

This success proves that we were initially right to select this approach and encourages us to continue in this powerful way to develop HPTLC as a relevant technique for our needs.

[1] Dubourdeaux, M. Procédé de Préparation d'extraits Végétaux Permettant l'obtention d'une Nouvelle Forme Galénique. 14 January 2009. Available at https://patents.google.com/patent/EP2080436A2/en, accessed on January 21, 2021)

[2] V. Bardot *et al.* (2020) Food Funct, https://doi.org/10.1039/c9fo01144g

[3] J.M. Roussel *et al.* (2021) J Chromtogr B, https://doi.org/10.1016/j.jchromb.2021.122923

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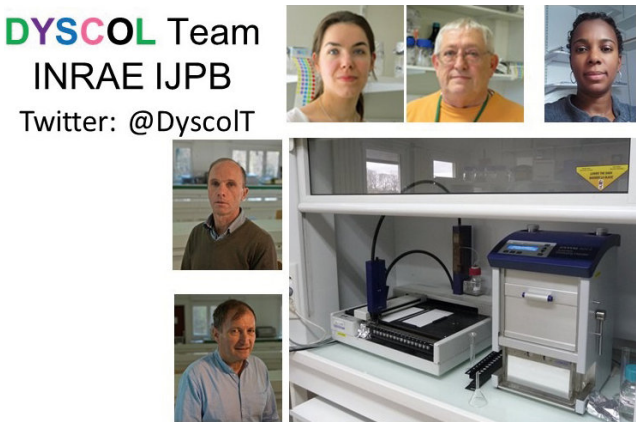
HPTLC – a useful tool for the characterization of enzymes from plant lipid metabolism



From lower left to upper right: Dr. Thierry Chardot, Prof. Pierre Briozzo, Dr. Laure Aymé, Michel Canonge, Christelle Louis-Mondésir

The Jean-Pierre Bourgin Institute (IJPB) is the largest center for plant biology at the National Research Institute for Agriculture, Food and Environment (INRAE). It combines resources and multi-disciplinary skills in the areas of biology, chemistry, and mathematics. Located in Versailles, it is a Joint Research Unit between INRAE and AgroParisTech. The DYSCOL (Dynamics and structure of Lipid Droplets) team studies various aspects of lipid accumulation in seeds from oil crops and model plants. The Kennedy pathway allows storage of fatty acids in eukaryotes in the form of triacylglycerols (TAG), through successive acylation of a glycerol backbone by acyltransferases. Hundreds of different fatty acids are found in plants. The different fatty acids incorporated into vegetable oils confer them specific physical, chemical and nutritional properties. DGATs (diacylglycerol acyltransferases) incorporate the final fatty acid in position *sn*-3 of the glycerol skeleton. They catalyze the rate-limiting step of the whole pathway. Due to their impact on oil yield and quality, DGATs are targets of interest for oil engineering. We aimed to identify candidate proteins as DGATs and to understand their substrate specificity. We used various approaches based on recombinant protein expression in different hosts, and analysis of the products of the reaction by complementary approaches, GC and HPTLC.

Introduction

Historically, demonstration of DGAT activity used radioactive precursors of substrates [1] followed by tedious extraction of the products (TAGs) [2] and quantification by liquid scintillation counting. Trans-methylation of TAGs produces fatty acid methyl esters, subsequently extracted, then separated and analyzed by GC. Alternatively, extraction of TAGs by improved methods and their separation from other cellular constituents by TLC is a convenient method avoiding radioactivity. Derivatization permits direct identification and quantification of the TAGs by comparison with standards.

HPTLC represents a valuable improvement of TLC. 12–15 samples are routinely separated at the same time on one plate. The method exhibits high sensitivity and reproducibility, and uses low amounts of organic solvents (< 50 mL for one run). Herein, we describe two methods to evidence DGAT activity and specificity using HPTLC.

Method (1) allows the analysis of extracted lipids by derivatization with phosphomolybdic acid reagent. Method (2) is used to study the activity and specificity of a purified DGAT.

Standard solutions

(1) Solutions of cholesteryl oleate, oleic acid methyl ester, trioleine, oleic acid, cholesterol at 2.7 µg/µL each in $CHCl_3$ are prepared.

(2) NBD-DOG (1-{N-[(7-nitro-2-1,3-benzoxadiazol-4-yl)-methyl] amino-decanoyl-2-decanoyl-*sn*-glycerol) stock solution in chloroform – methanol 2:1 (*V/V*) at 67.5 ng/µL (between 5.5 ng to 2.7 µg are applied to generate a calibration curve).

Sample preparation

(1) Lipids are extracted from yeast biomass according to Folch [2], dried under nitrogen, and re-suspended in 200 µL of chloroform – methanol 2:1 (*V/V*).

(2) DGAT assay to investigate enzyme specificity: NBD-DOG as a fluorescent DAG acceptor and different acyl donors (lauroyl-CoA, palmitoyl-CoA,

stearoyl-CoA, oleoyl-CoA, and linoleoyl-CoA. The reaction is carried out at 31 °C for one hour under shaking, then stopped by the addition of chloroform – methanol 2:1 (V/V).

Chromatogram layer

HPTLC plates silica gel 60 (Merck), pre-washed with isopropanol, are used.

Sample application

Between 3.0–6.0 µL for standard solutions and 50.0 µL for sample solutions (corresponding to 400 µg cells, dry weight) are applied as bands with the Automatic TLC Sampler (ATS 3), 15 tracks, band length 5.0 mm, distance from left edge 15.0 mm, distance from lower edge 8.0 mm.

Chromatography

Plates are developed in the Automatic Developing Chamber (ADC 2) with chamber saturation (with filter paper) for 20 min, (1) development with diethyl ether – hexane – methanol – acetic acid 60: 40:5:1 (*V/V*) to the migration distance of 80 mm (from the lower edge), drying for 20 min, (2) development with hexane – diethyl ether – acetic acid 80:20:2 (*V/V*) to the migration distance of 80 mm (from the lower edge), drying for 20 min.

Post-chromatographic derivatization

The plates are immersed in 5% phosphomolybdic acid in ethanol, then incubated for 30 min at 100 °C using an oven.

Documentation

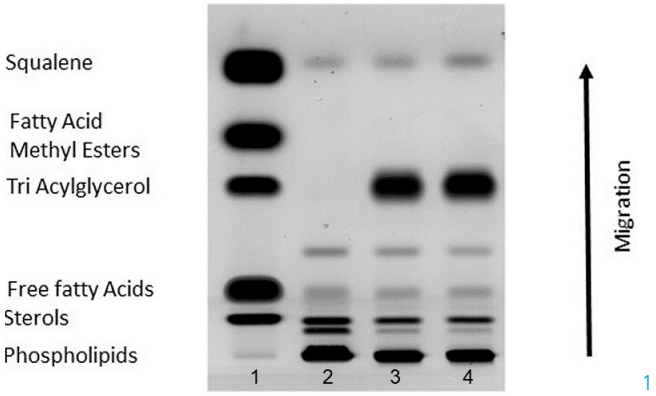
Images of the plates are captured in white light.

Densitometry

Fluorescence measurement is performed with the TLC Scanner 3 (excitation at 473 nm and emission > 510 nm). DGAT activity is expressed as picomoles of TAG formed per minute and per milligram of purified protein, using a calibration curve based on the fluorescent signal of NBD-DOG.

Results and discussion

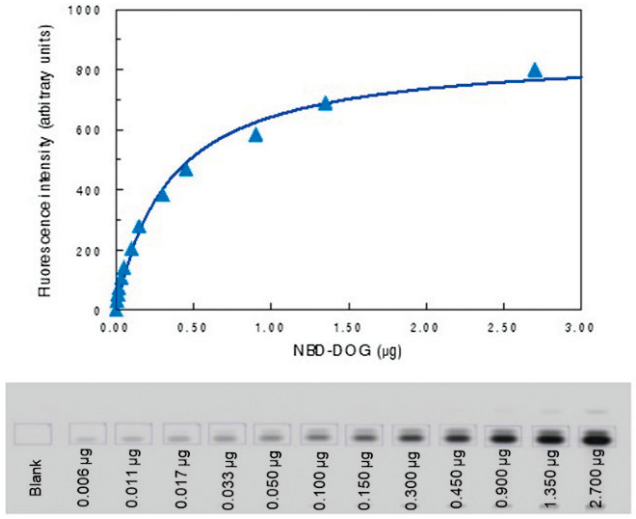
The following figure shows the results of the separation of lipids extracted from three yeast strains (method 1). The control yeast strain was transformed with an empty vector; a second strain was transformed with the AtDGAT1 sequence encoding for *Arabidopsis thaliana* DGAT1, and the last strain was transformed with the EgDGAT1 sequence encoding for *Elaeis guineensis* DGAT1-1, a putative DGAT [3]. Both cassettes encoding for plant DGAT1 restored TAG accumulation in the *Yarrowia lipolytica* mutant strain [4]. Thus, we conclude that EgDGAT1 sequence was encoding for an active *E. guineensis* DGAT1-1.



1

Separation of lipids extracted from yeasts strains expressing plant type 1 DGATs (1: standards, 2: empty cassette, 3: AtDGAT1, 4: EgDGAT1-1).

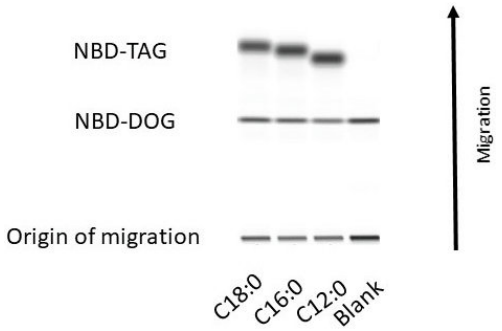
To generate a calibration curve (method 2), the fluorescence of the NBD-DOG standard applied in different amounts is measured. The standard curve is depicting the dependence of the intensity of the fluorescence of NBD-DOG (minus blank value).



2

Calibration curve of NBD-DOG (scanned at 473 nm in fluorescence mode) depicting the dependence of the intensity of the fluorescence of NBD-DOG (top) as function of the amount separated on HPTLC plate (bottom)

In another experiment, purified recombinant DGA1, a type 2 DGAT from the yeast *Yarrowia lipolytica* was incubated with NBD-DOG and acyl-CoA with various acyl chain lengths (C12:0, C16:0; C18:0).



3

Separation of TAG synthetized by purified recombinant yeast type 2 DGAT (DGA1) using acyl-CoA with different chain lengths.

In the absence of an acyl donor, no NBD-TAG was formed (blank track). In the presence of acyl donors (C12:0, C16:0; C18:0–CoA), NBD-TAGs were formed. Noticeably, the migration of the reaction product depended on the length of the acyl chain incorporated. NBD-TAG with C18:0 migrate over a longer distance by comparison to NBD-TAG with C16:0 or C12:0.

HPTLC is a fast (within two hours), reproducible, and robust method to evidence acyltransferases activities. *In vivo* (1), complementation of microbial strains affected in neutral lipid metabolism by sequences coding for DGAT led to accumulation of TAGs. The products were extracted, separated by HPTLC and identified directly by comparison with standard molecules. *In vitro* (2), DGATs transferred various acyls to fluorescent DAG acceptors. Fluorescent products of the reaction (TAGs) were quantified using a substrate calibration curve. The method was sensitive enough to distinguish TAGs differing by only two carbons [5]. Both approaches avoided the use of radioactive labeled products.

[1] Erickson, S.K. and Fielfing, P.E. J Lipid Res 27 (1986) 875–883
[2] Folch, J. *et al.* J Biol Chem 226 (1957) 497–509
[3] Aymé, L., *et al.* PLoS One (2015) 10: e0143113
[4] Beopoulos, A., *et al.* (2012) Appl Microbiol Biotechnol 93: 1523–1537
[5] Haili N., *et al.* (2016) J Biol Chem 277: 6478–6482

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4

HPTLC Online User Meetings, hosted by the HPTLC Association

Launched in spring 2022, the HPTLC Online User Meeting series facilitates the knowledge exchange between HPTLC users worldwide. With a total of 800 attendees, the first three meetings were a great success. The concept of highly interesting presentations from various application fields followed by a moderated interactive discussion with experts from the HPTLC Association, industry and academia turned out to be spot on.

Organized by the India Chapter of the HPTLC Association, the third HPTLC Online User Meeting was held on 19 September 2022 and covered the presentations "HPTLC and HPTLC-MS – An essential tool in the assessment of botanical dietary supplements" and "HPTLC – A good choice for extractables and leachables studies". To view the on-demand presentations of the first three HPTLC Online User Meetings, visit **https://bit.ly/3flK3V6**.

The fourth HPTLC Online User Meeting is organized by the Australia Chapter and will be held on 1 December 2022.

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