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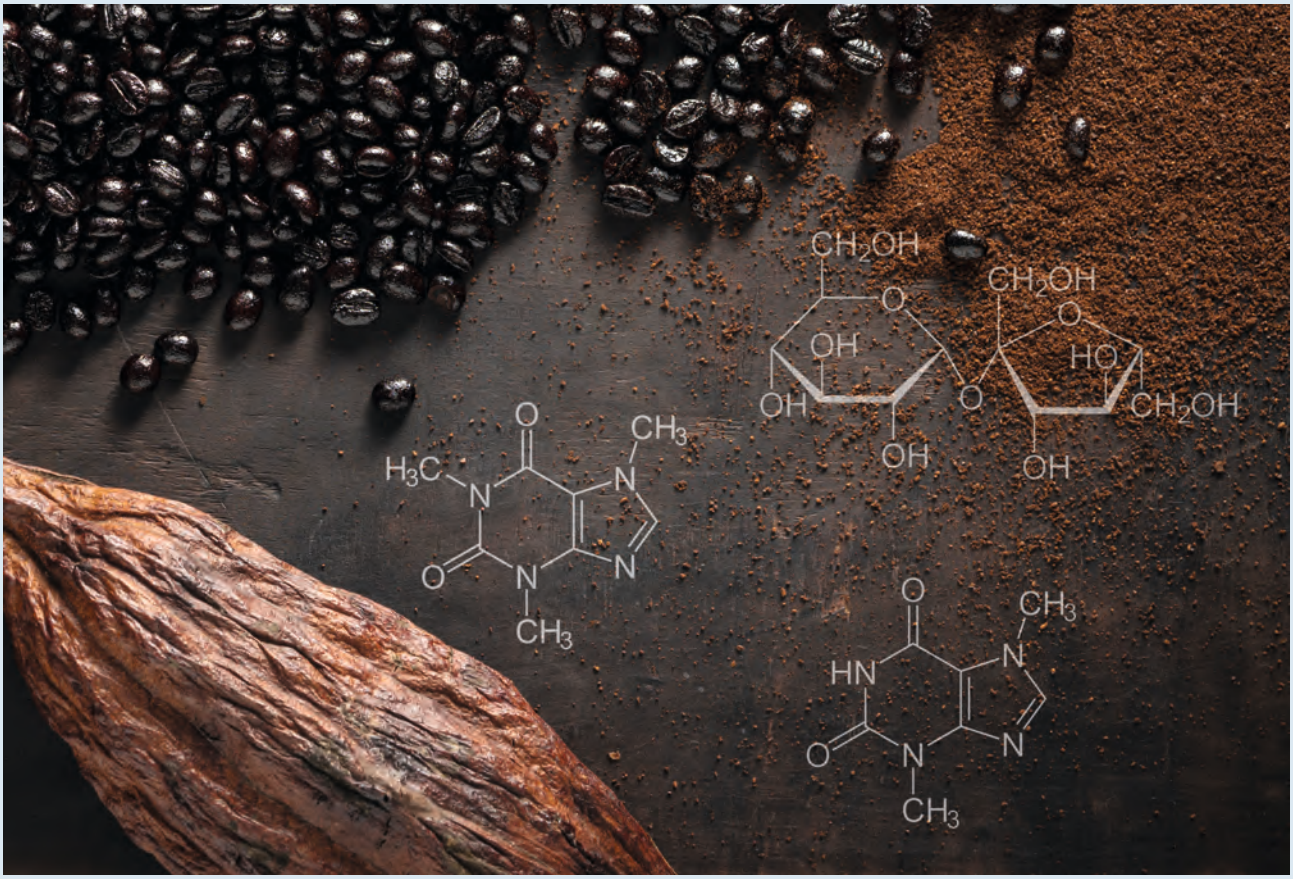

High-throughput analysis of aroma precursors in cocoa and coffee

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Fabry-related biomarkers in human plasma

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Untargeted Fabry-related Globotriaosylceramide (Gb_3) biomarkers in plasma

Left to right, standing: Dr. Luis Membrado, Dr. Carmen Jarne, Grad. José Manuel Escuín; sitting: Prof. Dr. Jesús Vela, Dr. Vicente L. Cebolla

The research group "Nanosensors and Bioanalytical Systems" consists of researchers from the Spanish National Center for Scientific Research (CSIC, at the Instituto de Carboquímica) and the University of Zaragoza. One of the group's areas of interest is the use of HPTLC-based techniques for analysis of complex mixtures mostly coming from petroleum conversion, biofuels and lipidomics. In addition to the inherent advantages of HPTLC (flexibility, complete sample detection, etc.), its coupling to any MS instrument through an elution-based interface opens up an unexplored range of analytical possibilities [1].

Introduction

Fabry disease is a severe genetic disease of lysosomal deposition produced by malfunction of an enzyme (α-galactosidase A), which results in a gradual accumulation of Gb_3, among other metabolites, in body fluids.

A number of Gb_3-related molecular species in the plasma of Fabry patients were detected at very low concentrations and demonstrated to be biomarkers, using reversed-phase LC coupled to mass spectrometry. HPTLC has generally not been considered as adequate for detailed characterization of a complex sample of lipids. This perception is not correct since currently HPTLC-MS is a methodology to get more information online in less time from complex biological matrices. HPTLC separation reduces mass spectral complexity, which, in turn, allows for a comprehensive examination of samples, even if incompletely resolved separations are obtained.

The objective of this work is to assess whether an HPTLC-densitometry-MS approach using the TLC-MS Interface 2 is adequate to determine Fabry biomarkers related to Gb_3 in human plasma.

A separation of a plasma extract into sphingolipid classes, followed by densitometry and MS coupling provides a simple but powerful approach for the detailed structural elucidation of sphingolipids present in human plasma, allowing their recognition by their *m/z*, and confirmation by their collision

induced dissociation MS/MS data, and/or APCI fragmentation.

The TLC-MS Interface 2 is suitable for the analysis of sphingolipids and provides a rapid, precise, and targeted characterization of selected bands on the plate. In our approach we use ESI-MS and APCI-MS. Intensities of ESI(+)-MS ions can be related to the concentration of species.

Samples and Standard solutions

Plasma samples from a Fabry's patient and a healthy control are obtained from the Institute of Health Sciences (Zaragoza, Spain) after approval of the Ethical Committee of Aragon (CEICA, Spain). Informed consent was obtained from the human subjects.

The investigated Gb_3 are untargeted species and no standards are available. Solutions of standards of lyso-Gb_3, Gb_3, lactosylceramide (LacCer), glucosylceramide (GlcCer) which represent the main sphingolipid families are prepared at a final concentration of 0.1 µg/µL each in dichloromethane – methanol 1:1 (*v/v*).

Sample preparation

Neutral sphingolipid extracts are obtained from plasma using a standard sample preparation procedure which involves centrifugation (10 min at 5000 rpm) to remove precipitated protein, followed by alkaline hydrolysis (75 µL of 2 M sodium hydroxide, incubated under magnetic stirring for 2 h at 40 °C), and liquid-liquid (H_2O-methanol) extraction. The lower layer containing the neutral sphingolipids is then transferred to a vial and dried under N_2. Samples are reconstituted in 250 µL of dichloromethane – methanol 1:1 (*v/v*).

Chromatogram layer

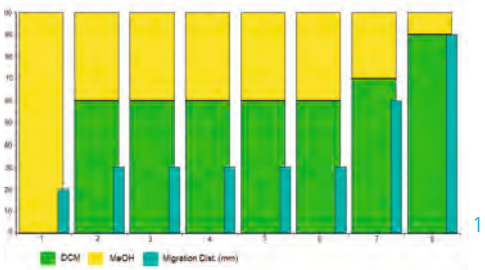
Two LiChrospher plates (20 x 10 cm, Merck) are used. They are pre-washed with methanol and kept in a desiccator in N_2 atmosphere until use.

Sample application

Sample and standard solutions are applied as 4 mm bands on the corresponding plate by using the Automatic TLC Sampler (ATS 4). Each of the plasma samples and standards are applied in triplicate. The distance between tracks is 10.6 mm, distances from the left and lower plate edges are 10 mm. One or more tracks are left empty, as blanks. Between 25.0–30.0 µL of plasma sample extracts and 0.1–10.0 µL of standard solutions are applied on the same plate.

Chromatography

Development is performed on two twin plates using the Automated Multiple Development (AMD 2) system under the conditions in the figure below.



1

AMD 2 gradient for plasma extract separation (final migration distance of 90 mm, DCM = dichloromethane, MeOH = methanol)

Post-chromatographic derivatization

One of the twin plates is immersed into 0.2 g of orcinol in 100 mL of 10% H_2SO_4 using the Chromatogram Immersion Device (immersion time: 2 s). The plate is heated during 15 min at 100 °C using the TLC Plate Heater 3.

Densitometry

Absorbance measurement is performed with the non-derivatized plate at 190 nm and with the derivatized plate at 550 nm using the TLC Scanner 3.

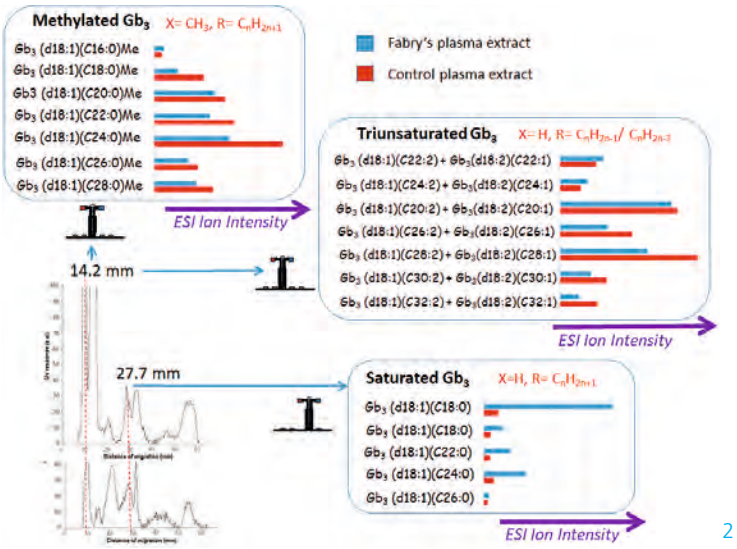
Mass spectrometry

Selected zones of the non-derivatized plate are eluted with the TLC-MS Interface 2 (oval head) at a flow rate of 0.2 mL/min with methanol into an ion-trap MS with ESI(+) ionization. Gb_3 species with *m/z* between 1000 and 1200 Da are scanned. HPTLC-ESI(+)-MS/MS is used for structure identification. Alternatively, APCI(+) ionization is used for structure confirmation by an analysis of derived fragments with *m/z* <1000.

Results and discussion

Identification and semi-quantification of 19 untargeted molecular species of globotriaosyl-ceramides (Gb_3) in extracts from a Fabry's plasma patient and a healthy control was performed by HPTLC-densitometry-MS. The species found were: five isoforms of saturated Gb_3, seven isoforms of methylated Gb_3, and seven species with three unsaturations (that of sphingosine plus two additional unsaturations). Twelve of these species were previously re-

ported as biomarkers of Fabry's lysosomal disorder using a LC-MS-based method, and the other seven are structurally similar, closely related to them [2].



2

Fabry's Gb3-related biomarkers and semi-quantitative ion abundance in Fabry (blue) and control (red) plasma extracts

Saturated Gb_3 isoforms come from the ESI(+)-MS analysis of the peak at 27.7 mm of Fabry extract (and 27.9 mm in the case of control). This is the migration distance of the Gb_3 standard. Ions are mostly detected as sodium adducts. The most preponderant isoform is d18:1;C16:0 (*m/z* 1046.8 $[M+Na]^+$). Confirmation of identity of this most abundant ion $[C_{52}H_{97}NO_{18}Na]^+$ is done by ESI-MS/MS by loss of a hexose group, giving a product ion at *m/z* 885.2.

Methylated and three-unsaturated Gb_3 species were both found in HPTLC-UV peaks at 14.9 (Fabry) and 14.2 mm (control). They should have *a priori* corresponded to preponderant sphingomyelin (SM) species. The obtained HPTLC-ESI(+)-MS showed intensities of 10^5 arbitrary units (a.u.), and high S/N ratio for SM species. However, we found that some Gb_3-related species are present in low concentration and can co-migrate together with the above SM species. Intensities for these Gb_3 species are 10^4 a.u. (arbitrary units).

The zone between *m/z* 1000-1300 displays ESI ions that matched with either methylated Gb_3-related isoforms as $[M+Na]^+$, or Gb_3-related isoforms or analogues with three unsaturations, as $[M+H]^+$. The presence of these structures found by ESI-MS was verified by an analysis of low-molecular ion fragments obtained using HPTLC-APCI(+)-MS [2]. This experiment was performed in duplicate and similar spectra were obtained in each case.

Ion intensities are related to concentration of Gb_3 species for several reasons summarized elsewhere [2]. Saturated Gb_3 in Fabry's plasma were in higher concentration than in control sample in repeated experiments. ESI-MS profiles for methylated and unsaturated Gb_3 species were qualitatively similar for Fabry and control samples although relative distribution of ions is different.

Conclusion

HPTLC-MS has been proven to be a simple but powerful approach for the detailed structural elucidation of sphingolipids present in human plasma. The potential of HPTLC-MS for lipidomic research in general has been summarized elsewhere [1].

[1] V.L. Cebolla *et al.* J Liq Chromatogr & Rel Technol (2021) https://doi.org/10.1080/10826076.2020.1866600

[2] C. Jarne *et al.* J Chromatogr A 1638 (2021) 461895

Further information is available on request from the authors.

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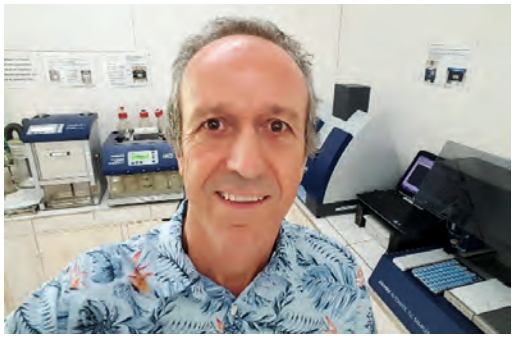
High-throughput analysis of aroma precursors in cocoa and coffee



Dr. Vincent Lebot

Dr. Vincent Lebot is a plant breeder and geneticist at CIRAD (Centre International de Coopération en Recherche Agronomique, France) and employs chromatographic separation techniques, especially instrumental planar chromatography, to select genetically improved tropical plant hybrids and varieties of suitable chemotypes. HPTLC is his preferred technique because of its low solvent consumption and running costs and its support of a high-sample throughput.

Introduction

It is estimated that there are around 24,000 varieties of cocoa and more than 30,000 varieties of coffee in the world [1, 2]. Only a few varieties have been evaluated for their quality aiming at specialty and aromatic products, which is an important trait for securing markets, especially for smallholders in small producing countries. During the fermentation and drying processes, aroma precursors present in the green beans are transformed into flavour compounds determining the quality of commercial beans. The chemical analysis of non-volatile compounds is, therefore, an essential step for the selection of varieties with desired quality traits. Alkaloids (theobromine and caffeine) and polyphenols (catechins, proanthocyanidins and anthocyanins) are contributing to the cocoa flavour. Caffeine, trigonelline and chlorogenic acids are known to produce astringent and sour taste and bitterness in coffee but very few varieties have been compared in controlled conditions. Sucrose is also an aroma precursor in coffee and depends on the variety. The objectives of this study were: i) to develop a protocol for the quantification of non-volatile compounds in cocoa and coffee, ii) to analyze and compare cocoa and coffee varieties from diverse geographical origins cultivated in controlled conditions and harvested at full maturity.

Standard solutions

Standard stock solutions of theobromine, trigonelline, caffeine, ideain-3-*O*-galactoside, cyanidin-3-*O*-arabinoside, (–)-epicatechin, (–)-catechin, chlorogenic acid (CGA), neochlorogenic acid (NCGA or 5-*O*-caffeoylquinic acid), 3,4-, 3,5-, 4,5-dicaffeoylquinic acids, and sucrose are prepared at 1.0 mg/mL with methanol. Standard solutions are prepared at different concentration levels with methanol and stored at 4 °C.

Sample preparation

Cocoa pods and coffee berries are handpicked and selectively harvested when they are completely mature to guarantee the complete uniformity of the material from the different accessions. All samples are collected from a single healthy tree per accession basis. Green beans of cocoa and coffee were then oven dried at 60 °C until constant weight (11% humidity) and milled. For each accession sample, 10 g of powder are mixed in centrifuge tubes with 30.0 mL of acetone for cocoa and 30.0 mL of methanol – water 7:3 (*v/v*) for coffee, sonicated for 10 min and then centrifuged at 1,585 x g for 10 min. The supernatant is transferred to a vial stored at 4 °C in the dark until analysis.

Chromatogram layer

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

Sample application

1.0 µL of standard and sample solutions are applied as bands with the Automatic TLC Sampler (ATS 4), band length 8.0 mm, distance from the left edge 15.0 mm, track distance 8.9 mm.

Chromatography

Plates are developed in the Automatic Developing Chamber (ADC 2) with ethyl acetate – toluene – formic acid – water 7:1:1:1 (*v/v*) without chamber saturation for cocoa extracts and with ethyl acetate – dichloromethane – formic acid – acetic acid – water 23:6:2:2:2 (*v/v*) with chamber saturation (10 min, with filter paper) for coffee extracts, to a migration distance of 70.0 mm. For sucrose, plates are developed with acetonitrile – water 87:13 (*v/v*) without chamber saturation to a migration distance of 85 mm.

Documentation

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

Densitometry

Absorbance measurement at 275 nm and 330 nm prior to derivatization and for sucrose at 520 nm after derivatization with TLC Scanner 4 and *winCATS*, slit dimension 8.00 mm × 0.20 mm, scanning speed 50 mm/s.

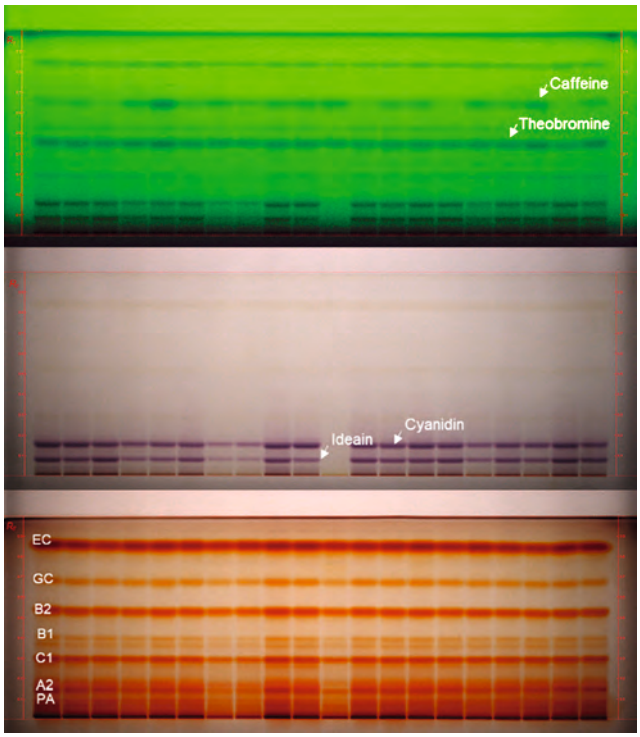
Editor's Note: A slit length of 5.00 mm is usually used for a band length of 8.00 mm.

Derivatization

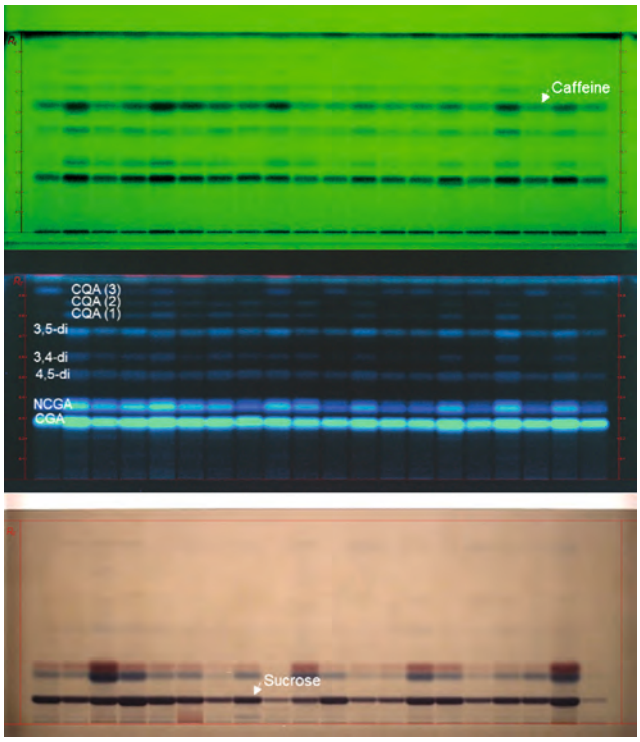
The plate is immersed into anisaldehyde reagent (for proanthocyanidins) or aniline-diphenylamine-phosphoric acid reagent (for sucrose) with the Chromatogram Immersion Device and heated on the TLC Plate Heater at 105 °C for 10 min.

Results and discussion

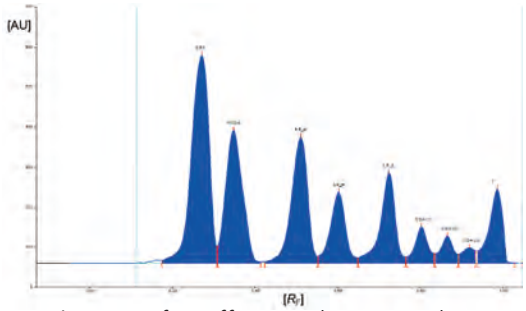
For repeatability accuracy assessment, linear ranges were computed using the least squares method. Repeatability was confirmed by applying four repetitions of each standard at five different concentration levels (0.1, 0.2, 0.3, 0.4, 0.5 µg/µL) and the variance among repetitions was expressed as the repeatability standard deviation (*%RSD*) [3]. Peak area measurements were compared to individual standards and corresponding values were quantified in mg/g dry weight [3]. The repeatability of the HPTLC measurements was assessed for each individual standard and the calibration plots were linear for all analytical standards with all $R^2 > 0.99$ ($P = 0.01$). For each compound, *%RSD* values were low (< 3.5%) indicating that the HPTLC measurements are accurate enough to be used for quantification.



1 *HPTLC chromatograms of different cocoa varieties (20 tracks, top: UV 254 nm prior to derivatization, middle: white light prior to derivatization, bottom: white light after derivatization with anisaldehyde reagent for polyphenols: (–)-epicatechin: EC and (–)-catechin: GC, and proanthocyanidins: PA, A2, C1, B1, B2 [3]*

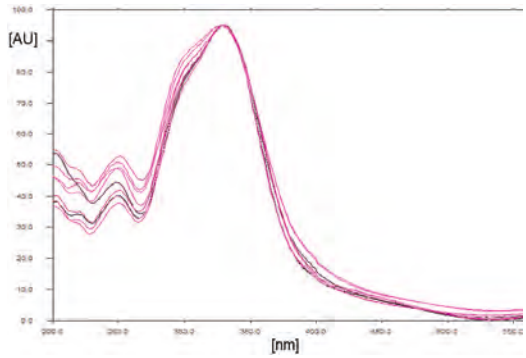


2 *HPTLC chromatograms of different Arabica coffee varieties (20 tracks, top: UV 254 nm prior to derivatization, middle: UV 366 nm prior to derivatization, bottom: white light after derivatization with ADPA reagent.*



3

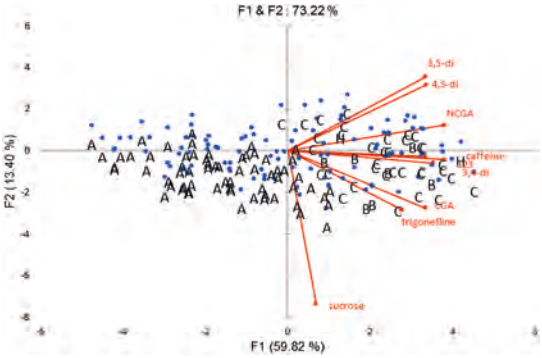
Densitogram of a coffee sample measured at 330 nm prior to derivatization (with increasing R_F values chlorogenic acid (CGA), neo-chlorogenic acid (NCGA or 5-O-caffeoylquinic acid), 4,5-dicaffeoylquinic acid (4,5-di), 3,4-dicaffeoylquinic acid (3,4-di), 3,5-dicaffeoylquinic acid (3,5-di), and three unknown caffeoylquinic acids labelled as CQA (1), CQA (2), CQA (3).



4

Overlay of UV spectra of CGA standard and samples (recorded from 200–550 nm) to determine the optimum wavelength for scanning densitometry (λ_{max} at 330 nm)

Multivariate analysis of non-volatile compounds (via peak areas obtained by scanning densitometry) in 137 cocoa varieties clearly discriminates the different groups of varieties (Amenolado, Criollo, Forastero, Trinitario). Theobromine was the most important compound in all accessions analyzed, followed by caffeine, epicatechin/catechin, cyanidin, and ideain. Proanthocyanidin B2 was also important and four other proanthocyanidins were minor compounds [3]. The 108 varieties of coffee analyzed were clearly differentiated based on their caffeine content which is significantly correlated with chlorogenic acids. Arabica varieties present low caffeine. Sucrose, trigonelline, caffeine, and eight chlorogenic acids were detected and quantified based on the R_F values of the standards and by matching their UV spectra with those of the samples [3]. Principal component analysis (PCA) of the coffee varieties shows that 73.2 % of the total variation is explained by axis 1 and 2. *C. arabica* are characterized by low alkaloids and CGAs contents and are mostly located on the left side of axis 1, while *C. canephora* characterized with high alkaloids and CGAs contents are located on the right side of axis 1. *C. arabica* with < 11 mg/g of caffeine are on the far left end of axis 2 and *C. canephora* with > 18 mg/g of caffeine are on the far right end of axis 2. Further results and details are available at [3].



5

PCA of 108 accessions of Coffee arabica (A), C. arabusta (B), C. canephora (C), and hybrids of C. canephora and C. congensis (H). The Arabica varieties (A) are differentiated from Canephora (robusta) varieties by their low chlorogenic acids contents (left of axis 1).

Conclusion

HPTLC is a cost-efficient technique when applied to both qualitative and quantitative assessment of chemical constituents in green cocoa and coffee beans. Compared to other chromatographic techniques, HPTLC presents the outcome as an image of the separated non-volatile compounds on a single plate, detected by UV light. This visible outcome and the simplicity of the technique could allow breeders to run the chromatographic procedure described in this study on hundreds or thousands of varieties and breeding lines. The analysis time for a plate with 20 tracks is comparatively short (approximately 38–42 minutes) and diverse genotypes can be analyzed side by side on the plate, making HPTLC the method of choice for rapid chemometric evaluation of cocoa and coffee varieties and detection of exceptional individuals.

[1] Laliberté, B. *et al.* (2012) https://agritrop.cirad.fr/568442/1/document_568442.pdf
[2] Bramel, P. *et al.* (2017) https://worldcoffeeresearch.org/media/documents/Coffee_Strategy_Low_Res.pdf
[3] Lebot, V. *et al.* Gen Res Crop Evol (2020) 67: 895–911

Further information is available on request from the author.

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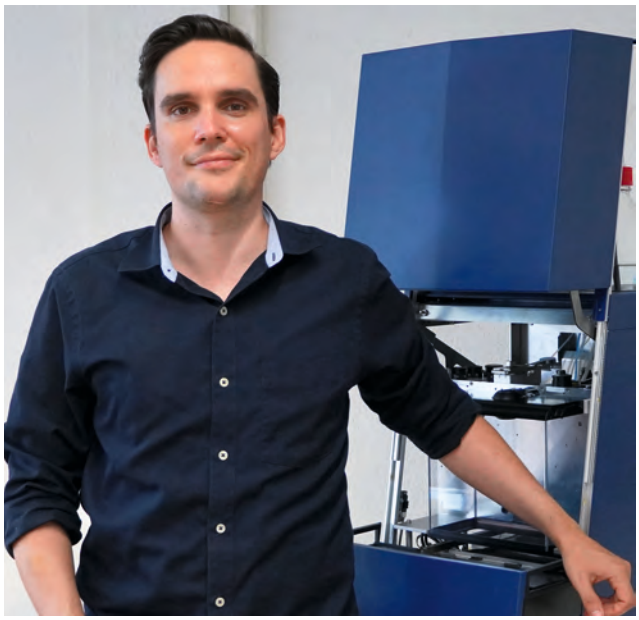
Product Management at CAMAG – managing the project HPTLC PRO Module DERIVATIZATION



I currently hold the position of Product Manager HPTLC at CAMAG. After finishing my studies in chemistry and nanotechnology, I did my Ph.D. in the Department of Microsystems Engineering at the University of Freiburg, Germany. For many years, I worked on the research and development of point-of-care devices, such as portable analyzers detecting toxins and pathogens from blood and environmental samples. After working in all major phases of product development, from research and prototype development to market introduction of devices and assays, I joined CAMAG in 2016.

The most recent project I have managed here is the HPTLC PRO Module DERIVATIZATION.

The focus of the Module DERIVATIZATION, as for the whole HPTLC PRO System, is the high-throughput analysis in routine quality control. The key innovations of the Module DERIVATIZATION are as follows:

To combine and automate all necessary derivatization steps

The module includes the main steps reagent transfer for derivatization and heat-treatment of the HPTLC plate to optimize the result. Integrating the possibility to choose between two connected derivatization reagents, to switch between up to three different nozzles types, and to automatically clean all relevant parts and nozzles with an optimized washing procedure offered quite a challenge to solve. The aerosol that remains after reagent transfer and potentially aggressive solvent gas created during heat-treatment are safely eliminated from the module.

Spraying of reagents at reduced pressure

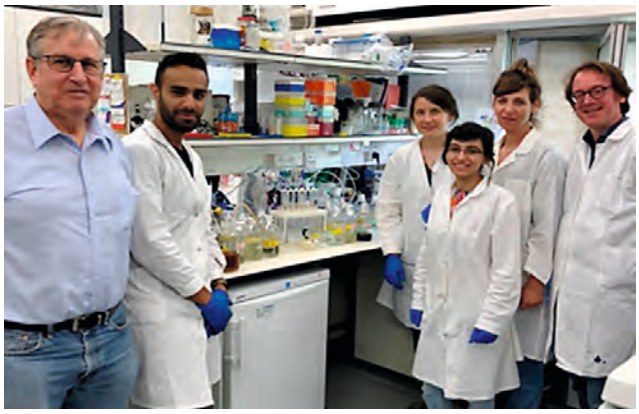
CAMAG's patented micro droplet spraying technology, which is already used in the stand-alone device Derivatizer, can be of limited use for viscous substances. Derivatization reagents might need heavy dilution or a change of solvent in order to be sprayed under atmospheric pressure. A new mode was introduced, which reduces the pressure in the spraying nozzle. This broadens the range of concentration and the choice of reagents, which can evenly be sprayed onto the HPTLC plate, and reduces the time required. Even more viscous reagents like ethanolic sulfuric acid can now be handled.

Tests with the Module DERIVATIZATION have shown additional benefits when applying reduced pressure during reagent transfer: the nozzle can form a more consistent reagent jet during spraying and additionally, nozzles that generate smaller droplets can be used, which leads to the best possible reagent distribution on the HPTLC plate.

CAMAG will officially introduce the Module DERIVATIZATION in early 2022. I am looking forward to launching the coming Modules DETECTION and MS-INTERFACE to complete the CAMAG HPTLC PRO SYSTEM and to help you use this new product at its best.

Dr. Thomas van Oordt
Product Manager

Parallel detection of estrogenicity and androgenicity using yeast-based fluorescent sensors



Left to right: Prof. Shimshon Belkin, Dror Shakibai, Carolin Riegraf, Nidaa Abu-Rmailah, Dr. Liat Moscovici, Dr. Sebastian Buchinger (missing in the photo: Hadas Atias and Georg Reifferscheid)

One research topic at the department of Biochemistry and Ecotoxicology of the German BfG (Federal Institute of Hydrology) is the development and improvement of effect-based methods for the rapid and cost-effective screening of environmental samples.

Researchers of the Life Sciences Institute at the Hebrew University of Jerusalem focus on the genetic engineering of whole-cell biosensors, the development of novel bioreporters and the directed evolution process for the improvement of sensor performance.

The collaboration between these two working groups is part of the German-Israeli cooperation in the water technology research project „Tracking Effects of Environmental organic micro-pollutants in the Subsurface“ (TREES), aiming at broadening the application of bioassays performed in combination with HPTLC.

Introduction

Endocrine disrupting compounds that enter the aquatic environment, *e.g.*, via wastewater treatment plant (WWTP) effluents, can affect the natural hormonal activity of freshwater organisms and thus pose a potential ecological threat even at low concentrations [1]. Prominent endocrine disrupting compounds bind to estrogen (ER) and androgen (AR) receptors, and thus trigger estrogenic and androgenic activity. These modes of action are detected with genetically engineered yeast cells, emitting a detectable fluorescence signal upon exposure to compounds exerting hormonal activity.

Following studies performed with *Arxula adeninivorans* yeast strains [2], the authors show here the parallel biological detection of estrogenicity and androgenicity in HPTLC-separated sample components using newly generated biosensors based on *Saccharomyces cerevisiae*. These biosensors express human estrogen and androgen receptors, co-transfected with respective reporting elements that produce either the red fluorescent protein mRuby2 in response to an estrogenic stimulus (ER-Ruby) or the blue fluorescent protein mTagBFP2 in response to an androgenic stimulus (AR-BFP).

The advantage of using HPTLC lies in its biocompatibility, which allows the combination of sample fractionation with biological effect detection directly on HPTLC plates. Several samples can be screened for estrogenicity and androgenicity on parallel tracks simultaneously, thus reducing time and effort. Its matrix robustness allows the assessment of samples with complex and heavy matrices such as WWTP influents.

Standard solutions

Stock solutions of testosterone (0.5 mg/mL) and dihydrotestosterone (DHT, 5 mg/mL) are prepared in ethanol as androgenic reference compounds. Estrone (E1), 17ß-estradiol (E2) and estriol (E3) in ethanolic stock solutions of 5 mg/mL serve as estrogenic reference compounds. An androgenic and an estrogenic mixture was prepared and diluted depending on range-finding tests.

Sample preparation

Influent and effluent grab samples of two different municipal WWTP are enriched 200x and 500x by solid-phase extraction, respectively.

Chromatogram layer

HPTLC plates silica gel 60 F_{254} (Merck), 20 x 10 cm, are used. Plates are pre-developed with 100% methanol to 95 mm, dried in an oven at 120 °C, and then stored in a desiccator at room temperature.

Sample application

The application of samples and standard solutions as bands is performed with the Automatic TLC Sampler (ATS 4): up to 11 tracks, band length 5.0 mm, distance from left edge 20.0 mm, track distance 16.0 mm, distance from lower edge 8.0 mm, application volume 10.0 to 20.0 µL for sample solutions and 5.0 to 10.0 µL for standard solutions.

Chromatography

HPTLC plates are developed in the Automated Multiple Development System (AMD 2). A focusing step is performed with 100% methanol to a migration distance of 20 mm, followed by drying for 2 min. In a second step, the development to a migration distance of 90 mm is conducted with ethyl acetate – *n*-hexane 1:1 (*v/v*) followed by drying for 5 min.

Yeast biosensors

For the planar bioassay, a yeast co-culture is prepared by mixing AR-BFP and ER-Ruby yeast cell cultures, previously adjusted separately to 1000 ± 50 formazine attenuation units. Then, 3.0 mL of this yeast co-culture are sprayed onto HPTLC plates using the Derivatizer (nozzle: yellow, level: 5). Plates are incubated at 30 °C for 18 h.

Documentation

Images are captured in UV 366 nm and white light with the TLC Visualizer.

Densitometry

Fluorescence measurement is performed with TLC Scanner 4 and *visionCATS* (slit dimension 10.0 x 0.6 mm and scanning speed 20 mm/s). The slit-dimensions reported in Schoenborn *et al*. (2017) [3] were used for these experiments; however, a further optimization of the slit dimensions under consideration of the signal broadening might improve the results. For detection of estrogenicity (ER-Ruby), excitation wavelength λ_{ex} = 525 nm and a K540 nm filter is applied. The detection of androgenicity (AR-BFP) is performed using an excitation wavelength λ_{ex} = 396 nm and a K400 nm filter.

Results and discussion

The aim of the presented study was to generate yeast-based fluorescent biosensors for the parallel detection of estrogenicity and androgenicity. In contrast to yeast-based biosensors using the expression of LacZ as reporting element, which requires the disruption of the cell wall and the addition of an external substrate [3], the presented biosensors allow a fast signal detection in living cells.

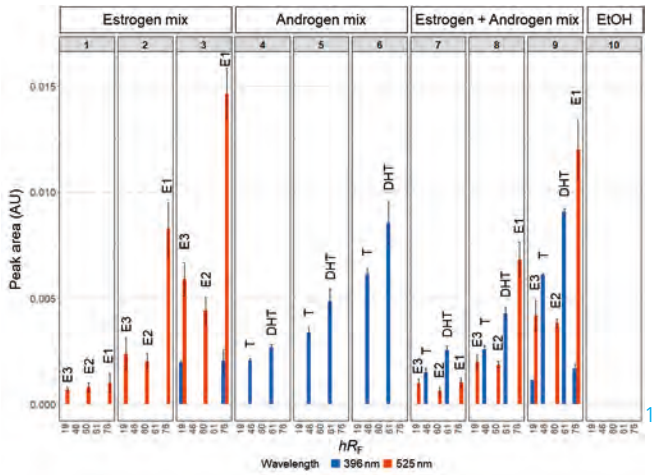
The general functionality of the individual yeast biosensors was assessed in 96-well plates and in combination with HPTLC, using either individual reference compounds or their mixtures [4].

Then, the combination of the two yeast biosensors as co-culture was investigated, using three dilutions of an estrogen mix of E1, E2 and E3 and an androgen mix of testosterone and DHT. The estrogen mix was applied on tracks 1–3, the androgen mix on tracks 4–6, and both mixtures were applied on tracks 7–9. Ethanol served as control on track 10. A distinction of the different hormonal activities in the same test can be made by changing the excitation wavelength for scanning. The repeatability was determined by calculating the standard error of minimum three replicates performed on different days, using separate aliquots of the cell suspension.

Dose-effect relationships of the estrogenic and androgenic components in the mixtures were detected. The ER-Ruby biosensor detects only estrogenic compounds (red), even in the presence of androgenic compounds, highlighting the specificity of this biosensor. A slight decrease in the signal intensity at λ_{ex} = 525 nm was detected, when estrogenic and androgenic model compounds were applied in a mixture. This suggests a weaker expression of the Ruby protein under these conditions.

Regarding the AR-BFP strain, the signal intensity of androgenic compounds (blue) remains unchanged in the presence of estrogenic compounds. However, some interference at the hR_F values specific for E1 and E3 is observed in case of high concentrations of the reference compounds. These signals were also detectable when only the ER-

Ruby biosensor was applied [4]. Possibly, high concentrations of the Ruby protein lead to detectable excitation of Ruby at λ_{ex} = 396 nm. On the other hand, the used lamps have a low output at 396 nm, which is as well very close to the cut-off filter at 400 nm. In any case, the optical system has to be refined by optimized filters used for the scanning, which might help avoiding this artefact in the future.



1

Applicability of a yeast co-culture for parallel detection of estrogenicity and androgenicity in model compound mixtures by fluorescence scanning at λ_{ex} = 396 nm (blue) and λ_{ex} = 525 nm (red). Estrogen mix consisting of E1 (0.01 ng, 0.05 ng and 0.1 ng), E2 (0.005 ng, 0.01 ng and 0.02 ng), and E3 (0.5 ng, 1 ng and 2 ng) was applied on tracks 1–3 and 7–9. Androgen mix consisting of testosterone (T; 0.5 ng, 1.0 ng and 5.0 ng) and DHT (0.5 ng, 1.0 ng and 5.0 ng) was applied on tracks 4–6 and 7–9. Reproduced with modifications from [4] (https://creativecommons.org/licenses/by/4.0/legalcode).

An example of the yeast-based biosensor application for a simultaneous screening for androgenicity and estrogenicity using influent samples from a municipal WWTP is shown below. Matrix components are observable below the focusing line and are thus well separated from active compounds. Both influent samples showed estrogenicity and androgenicity. Due to the similar structures and physico-chemical properties of estrogens and androgens, a separation of the two compound classes by HPTLC is challenging [2]. However, the multi-parallel effect assessment in combination with HPTLC allows the detection of the different effects even if the active compounds are not fully separated. In the presented WWTP influents, two strong estrogenic and androgenic signals overlap at hR_F values of 75 and 61, indicating the possible presence of E1 (hR_F = 76),

2

The Self-Education Platform – HPTLC Training by the International HPTLC Association

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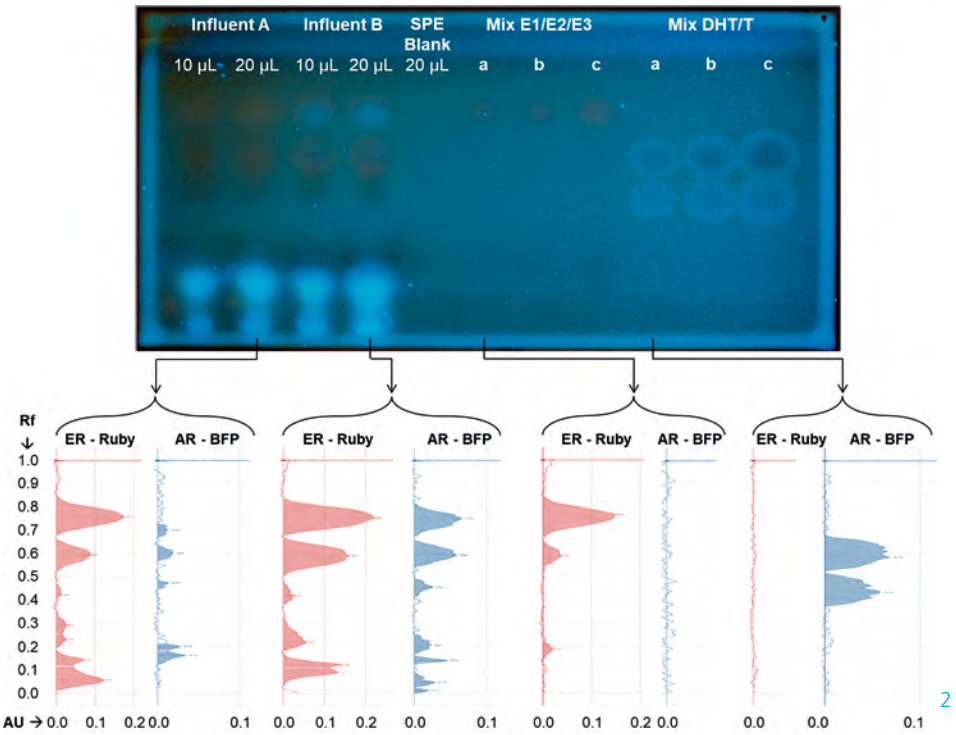
The Association's mission is to promote the use of HPTLC in plant analysis and other analytical fields and to bring together representatives from academia, industry, research, regulatory and standard-setting bodies.

Further information:
https://selfeducation.ch and
www.hptlc-association.org

E2 (hR_F = 60) and DHT (hR_F = 61). For the androgenic band at hR_F = 75, no candidate compound can be assigned. At some positions, signals from the ER-Ruby strain are visible between the tracks. Due to the leakiness of promoters, each reporter strain produces a background signal, which might be detectable especially at positions where cell densities are higher due to an inhomogeneous application of cells. However, an automated spraying device (Derivatizer) was used resulting in an even application of the cells. A further possibility is a signal broadening that can happen when silica-plates are used.

In conclusion, the presented study showed a proof of principle of the parallel detection of estrogenic and androgenic effects using a fluorescent yeast-based biosensor co-culture.



Parallel detection of estrogenicity and androgenicity in wastewater treatment plant influents using a yeast-based biosensor co-culture of ER-Ruby (red, λ_{ex} = 525 nm) and AR-BFP (blue, λ_{ex} = 396 nm). Estrogen mix: E1 (a: 0.1, b: 0.2 and c: 0.4 ng), E2 (0, 20 and 40 pg) and E3 (1, 2 and 4 ng); androgen mix: DHT (a: 10, b: 25 and c: 50 ng) and testosterone (T; a: 10, b: 25 and c: 50 ng). Top: plate image displaying the fluorescent signal enhanced using the enhancement tool of the visionCATS software. Reproduced with modifications from [4] (https://creativecommons.org/licenses/by/4.0/legalcode).

[1] A.P.A. Da Silva *et al.* Aquat. Sci. Technol (2018) 6: 35-51.
[2] A. Chamas *et al.* Sci. Total Environ. (2017) 605–606:507–513.
[3] A. Schoenborn *et al.* J Chromatogr A (2017) 1530:185–91.
[4] L. Moscovici *et al.* Biosensors (2020) 10(11): 169.

Further information is available on request from the authors.

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Use of *visionCATS* and quantified Reference Extracts (qRE) for qualitative and quantitative evaluations of herbal material

Left to right: Dr. Ophélie Fadel (Chromacim), Dr. Debora Frommenwiler (CAMAG), Dr. Daniel Jean (ISV), Dr. René de Vaumas (Extrasynthese)

Dr. Daniel Jean is the founder and director of ISV (*Institut des Substances Végétales*, France), which produces the quantified Reference Extracts (qRE) used in this study. Dr. Ophélie Fadel is the head of the application laboratory of Chromacim (France). Dr. Debora Frommenwiler is scientist at CAMAG (Switzerland), and Dr. René De Vaumas is the CEO of Extrasynthese (France), the distributor of qRE.

Introduction

Quality control of herbal drugs, extracts and products is a challenging task because it requires proper identification and determination of content of active compounds or markers. Because monographs of the pharmacopoeias typically require independent methods for these tasks, the cost of analysis may be quite high. Reference materials are a major contribution to the cost.

Quantified Reference Extracts (qRE) may be considered as suitable reference material in this context, reducing the need for stocking several separate chemical reference substances.

The goal of this work was to elaborate examples for using qRE for identification of plant materials and quantification of markers.

Standard solutions

For ginkgo, 0.2 mg/mL each of rutin and quercetin are prepared in methanol. The qRE *Ginkgo biloba* leaves is prepared at 30 mg/mL in methanol. For olive leaf, oleuropein is prepared at 1.0 mg/mL in methanol. For rosemary, rosmarinic acid is prepared at 0.25 mg/mL in ethanol, and the qRE *Rosmarinus officinalis* leaves at 1.0 mg/mL in 50% ethanol.

Sample preparation

1.0 g of powdered ginkgo leaf is mixed with 10 mL of methanol and refluxed in a water bath for 10 minutes. The mixture is centrifuged and the supernatant is used as test solution. The rosemary dried extract is prepared at 1.0 mg/mL in methanol. The qRE *Olea europaea* leaves (used as a sample) is prepared at 1.0 mg/mL.

Chromatogram layer

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

Sample application

Automatic TLC Sampler (ATS 4), application as bands, 15 tracks, band length 8.0 mm, distance from left edge 20.0 mm, distance from lower edge 8.0 mm. For the ginkgo project, application volume of 3.0 µL for sample solutions and for standard solutions. For olive leave different volumes of the oleuropein standard and 10.0 µL of the qRE were applied. For rosemary, the solution of the qRE was applied with different application volumes, and the rosemary dried extract was applied with 10.0 µL.

Chromatography

In the ADC 2 with 20 min chamber saturation (with saturation pad) and after activation of the plate at 33% relative humidity for 10 min using a saturated solution of magnesium chloride. The developing distance is 70.0 mm (from the lower edge). Plates are dried for 5 min. Ethyl acetate – formic acid – acetic acid – water 100:11:11:26 (*v/v*) is used as developing solvent.

Post chromatographic derivatization

For rosemary and ginkgo, the plates are heated using the TLC Plate Heater (100 °C, 3 min), then derivatized with NP reagent (1.0 g 2-aminoethyl diphenylborinate in 100 mL methanol) using the Derivatizer (3.0 mL, nozzle: green, level: 4). For olive leaf, the plate is derivatized with anisaldehyde (AS) reagent (0.5 mL of *p*-anisaldehyde dissolved in

85 mL methanol, 10 mL acetic acid, and 5 mL sulfuric acid) using the Derivatizer (3.0 mL, nozzle: blue, level: 3). The plate is heated at 100 °C for 3 min on the TLC Plate Heater.

Documentation

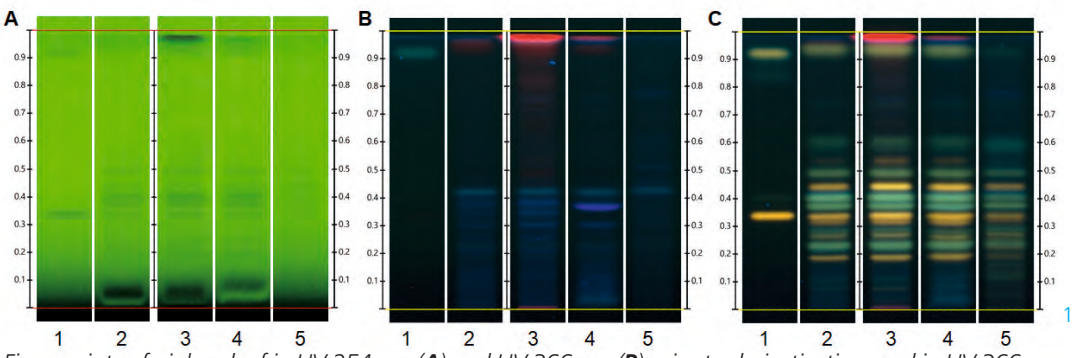
TLC Visualizer in UV 254 nm, UV 366 nm, and white light prior to derivatization, and UV 366 nm, and white light after derivatization.

Densitometry

TLC Scanner 4 and *visionCATS*, absorbance measurement at 254 nm and 238 nm, slit dimension 5.00 mm x 0.20 mm, scanning speed 50 mm/s, for oleuropein and in fluorescence mode at 366>/400 nm for rosmarinic acid.

Results and discussion

The first part of the project demonstrates the suitability of the ginkgo quantified Reference Extract as a standard for identification. The fingerprint of the qRE was compared to a reference sample of powdered ginkgo leaf extracted with methanol (track 3) or with 50% ethanol (track 4), respectively, and a market sample of ginkgo leaf dried extract (track 5). The fingerprint of the qRE resembles those of the corresponding herbal drugs and dried extract.



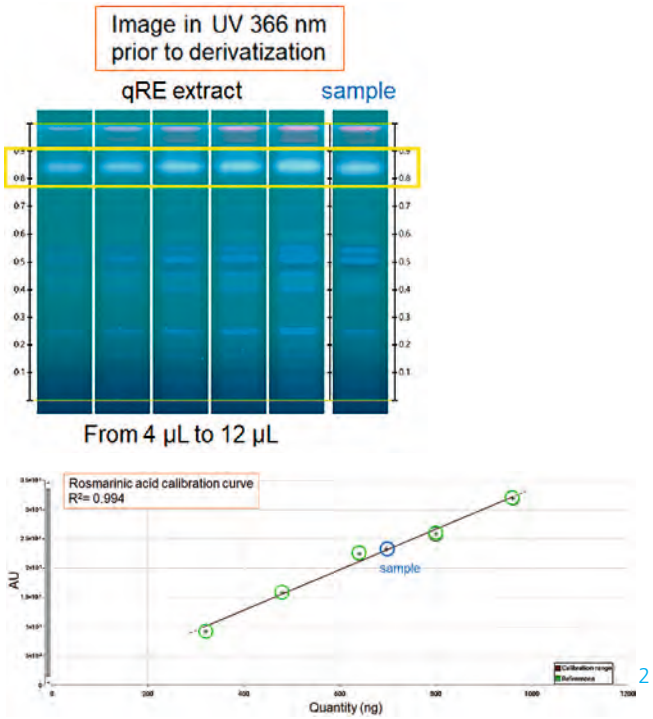
*Fingerprints of ginkgo leaf in UV 254 nm (**A**) and UV 366 nm (**B**) prior to derivatization, and in UV 366 nm after derivatization (**C**). Track 1: rutin, quercetin (with increasing R_F); 2: qRE extract; 3–4: powdered leaf; 5: dry extract.*

The second part of the study concerned the verification of the assigned content of oleuropein and rosmarinic acid in their respective qRE by HPTLC. Assigned contents, given in the qRE's certificate of analysis, were initially determined by HPLC. Chemical reference substances were used for quantification, and different detection modes were compared. Comparable contents were found for both markers.

Quantification of oleuropein and rosmarinic acid in the qRE extracts

	Detection mode	Derivatization	HPTLC		Assigned content	
			% w/w	CV (%) (*n*=3)	% w/w	CV (%)
Oleuropein	254 nm (image)	Prior to	23.1	0.00	23.47	4.5
	254 nm (scan)	Prior to	21.8	2.54		
	238 nm (scan)	Prior to	24.1	1.10		
	238 nm (scan)	After AS	23.2	1.60		
Rosmarinic acid	366 nm (image)	Prior to	8.4	3.22	8.64	4.5
	366 nm (image)	After NP	7.5	8.80		
	366 nm (scan)	After NP	7.9	4.32		

The third part of the study used one qRE and its assigned content of rosmarinic acid as reference for the quantification of rosmarinic acid in a rosemary dried extract. A solution of the qRE was applied with different application volumes to give a linear calibration curve. The content of rosmarinic acid in the sample was determined at 6.3 %.



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Fingerprints of the qRE calibration curve and the sample. Top: image in UV 366 nm; bottom: calibration curve

Conclusion

These examples show that qRE work well for the identification of plant materials and quantification of markers by HPTLC. For use in quality control, methods may need to be adapted from pharmacopoeia monographs.

Further information is available on request from the authors.

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3

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Sample application is the first step in the workflow of TLC/HPTLC. It significantly affects the quality of the results at the end of the process. It is a key factor for high precision and productivity in routine analysis.

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1

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