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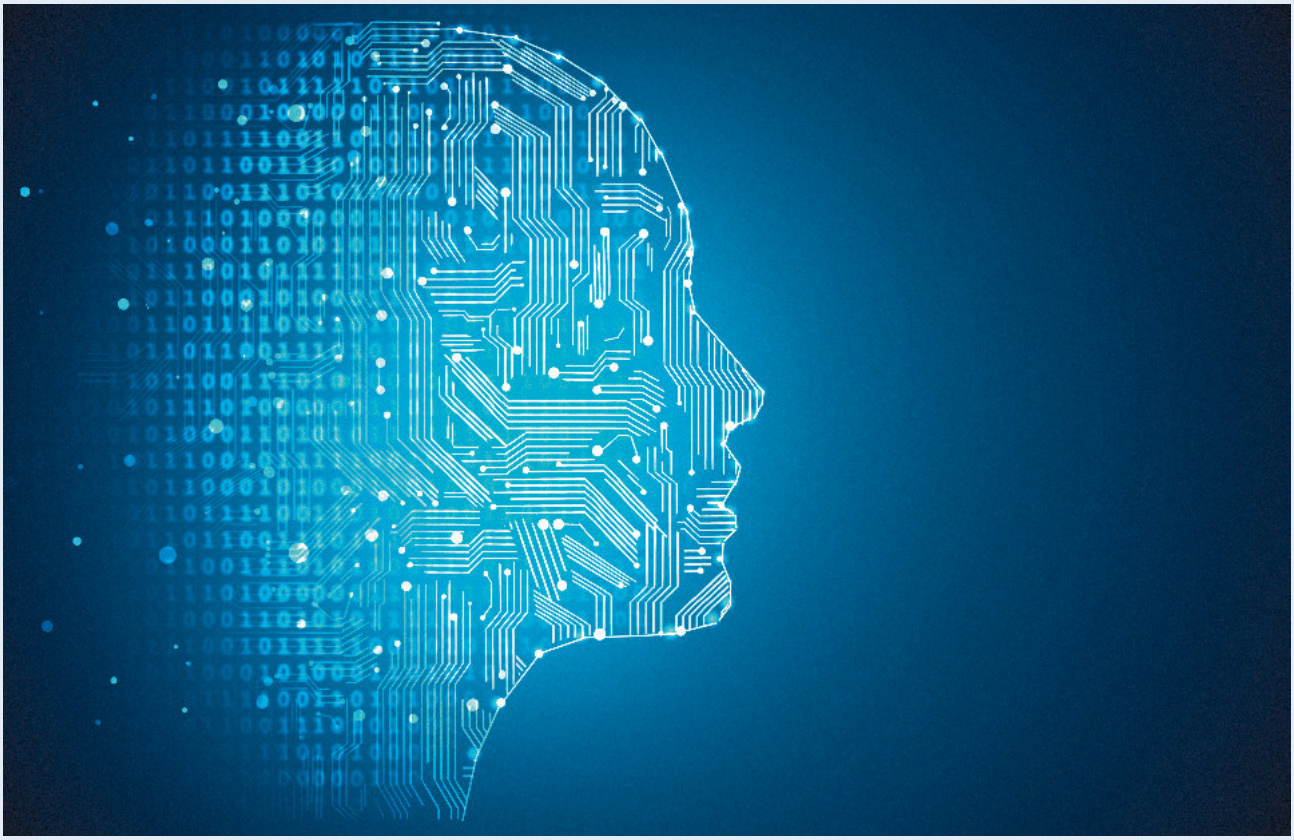

Use of machine learning to predict the R_F values

Featured articles:

- Extractables and leachables studies
- Analysis of 5-hydroxy-methylfurfural
- Stability testing of dihydroartemisinin
- HPTLC fingerprinting of fructooligosaccharides

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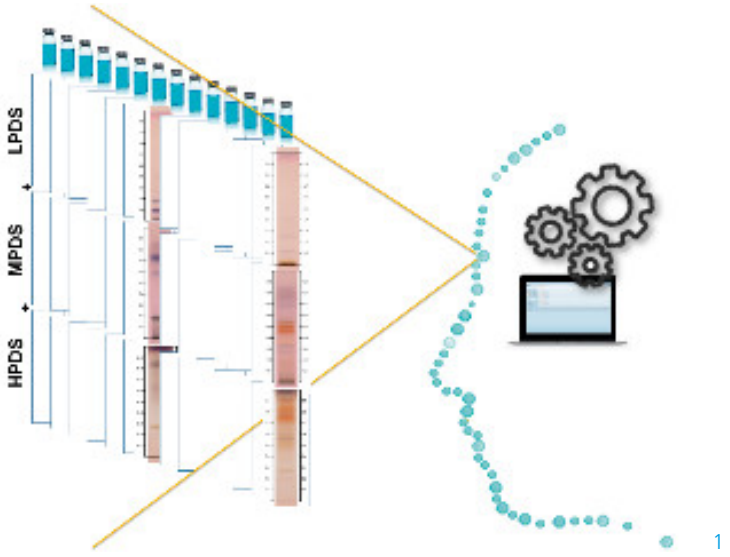
Applying machine learning to data obtained with the complementary developing solvents protocol

Introduction

In 2022, CAMAG laboratory introduced the concept of complementary developing solvents (CDS) based on the combination of three automated analyses using three developing solvents (DS): one low polarity (LPDS), one medium polarity (MPDS), and one high polarity (HPDS) solvent [1]. With these three DS, any compound is characterized by three R_F values instead of one. Introduced into a database, a large dataset can be compiled from these values, which then may be subjected to data mining and machine learning.

In their paper [2], CAMAG researchers describe how the application of a CDS protocol to a large number of highly diverse individual compounds was used in combination with machine learning to predict the R_F values of individual substances from molecular properties, and to generate proposals for the identity of a zone. Coupled with machine learning, the CDS concept as a very powerful, general, and medium to high throughput technique for routine analysis and sophisticated research, may become the future of HPTLC. It may help replacing common tasks such as visual evaluation and pattern recognition, as well as subjective pass/fail decisions by automated procedures and numerical values generated by suitable algorithms.



Visualization of the CDS and its composite fingerprint 1

Standard solutions

Individual standard solutions were prepared at a concentration of 1.0 mg/mL (adjusted when necessary). Methanol was used as solvent for iridoids, coumarins, pharmaceutical drugs, flavonoids, triterpenes, sesquiterpenes, steroids, phospholipids and cannabinoids, 50% aqueous acetonitrile for carbohydrates, 50% aqueous methanol for amino acids, and toluene for monoterpenes. System Suitability Test (SST): the ready to use solution of Universal HPTLC Mix (UHM) was prepared in house according to [3] and applied on each plate.

Chromatogram layer

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

Sample application

2.0 µL of sample solutions 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 (toluene, ethyl acetate 9:1 (*V/V*)) is used without saturation, whereas MPDS (cyclopentyl methyl ether, tetrahydrofuran, water, formic acid 40:24:1:1 (*V/V*)) and HPDS (ethanol, dichloromethane, water, formic acid 16:16:4:1 (*V/V*)) are used with 20 min chamber saturation (with saturation pad). The developing distance for all three methods was 70 mm (from the lower edge). Plates were dried for 5 min.

Post-chromatographic derivatization

Derivatization with anisaldehyde sulfuric acid reagent (10.0 mL of sulfuric acid were carefully added to the ice-cold mixture of 170.0 mL of methanol and 20.0 mL of acetic acid. To this solution, 1.0 mL of anisaldehyde was added) by spraying (Derivatizer, blue nozzle, 3.0 mL, spraying level 3) was followed by 3 min of heating at 100 °C. Images of plates derivatized with Fast blue salt B reagent (250.0 mg of Fast blue salt B (*o*-dianisidine bis(diazotized) zinc double salt) were dissolved in 10.0 mL of water and mixed with 25.0 mL of methanol and 15.0 mL of dichloromethane) were captured within 2 min after spraying (Derivatizer, green nozzle, 3.0 mL, spraying level 5).

For the derivatization with NP reagent (1.0 g of diphenylborinic acid aminoethylester was dissolved in 200 mL of ethyl acetate) / PEG (10 g of polyethylene glycol 400 (macrogol) were dissolved in 200 mL of dichloromethane), the plates were heated at 100 °C for 3 min, cooled to room temperature, then sprayed with the mixture NP/PEG 1:1 (*V/V*) (Derivatizer, green nozzle, 3.0 mL, spraying level 3), and dried for 2 min. Derivatization by immersion (Immersion Device, speed 5, time 0) with toluene sulfonic acid reagent (10% of *p*-toluene sulfonic acid in ethanol) was followed by heating at 150 °C for 3 min.

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 (as needed).

Densitometry

For the UHM, TLC Scanner 4 and *visionCATS*, absorbance measurement at 254 nm, slit dimension 5.00 mm x 0.20 mm, scanning speed 50 mm/s, and in fluorescence mode at 366>/400 nm.

Numerical databases preparation and processing

The open-source software KNIME (version 4.6) was used. The "RDKit KNIME Integration" was applied for curation of the databases and conversion of the chemical structures. 178 chemicals of the learning set were then used to benchmark various machine learning models.

Machine learning

R_F values obtained from peak profiles from images (PPI) or scanning densitometry (PPSD), were used with the Random Forest regressor algorithms including 100 trees.

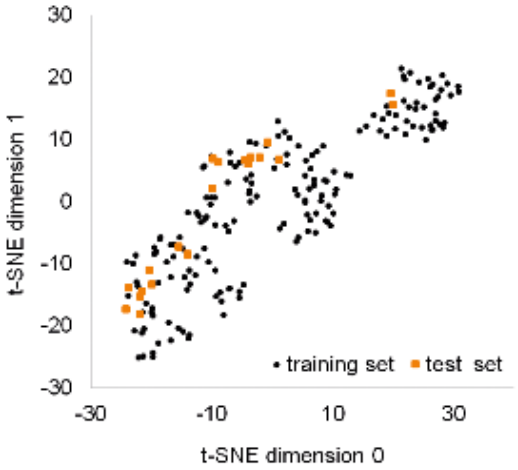
Results and discussion

For building a powerful model, four steps were taken:

2 *Overview of the machine learning pipeline and its workflow*

The first step was the collection of data. For this, a training set consisting of 178 known individual substances was selected from various chemical classes, covering molecular weights (MW) ranging from 75.1 g/mol to 1131.3 g/mol, and computed octanol/water partition coefficients (SlogP) in the range of -7.53 to 13.98. Using the open source software KNIME and its extensions, molecular descriptors (*e.g.* MW, SlogP, topological polar surface area (TPSA) …) were computed for each substance. In addition, each substance was chromatographed with the CDS, generating 178 x 3 = 534 R_F values. In the second step, the dataset was cleaned by filtering all descriptors for null variance. The third step included the training of the model. For this, the performance of three regressors was evaluated according to their capacity to predict the R_F within the training set. The Random Forest, trained with 100 trees, yielded the best correlation coefficients R^2 0.55, 0.72, and 0.64 for the LPDS, the MPDS, and the HPDS, respectively.

For testing of the model, a test set was created with 20 other substances. The suitability of the selected substances was verified by demonstrating that the chemical space of the test set was within the chemical space of the training set.

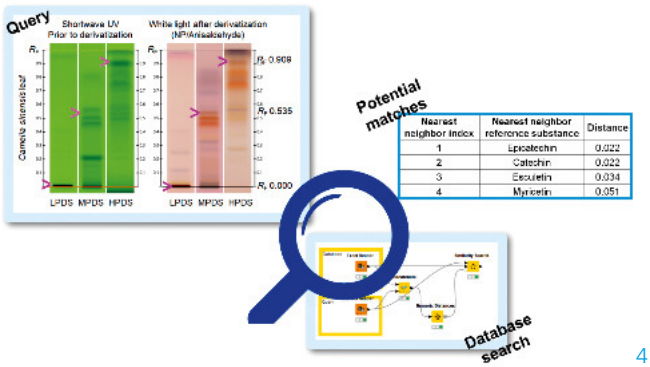


3 *Chemical space (2D t-SNE projection) covered by the 178 chemicals of training set (black dots) and the 20 chemicals belonging to the test set (orange squares).*

The model was used to predict the R_F values of the compounds in the test set. Most predicted R_F differ by less than 0.1 units from the measured values. R_F in the MPDS and the HPDS are both predicted within the correct range and with very small errors, leading to R^2 of 0.87, and 0.71, respectively. The variance for each individual prediction (LPDS, MPDS, and HPDS) remains smaller than 10%, except for a few compounds.

	Reference substance	R_F	Pred	R_F	Pred	R_F	Pred
1	Esculin	0.002	0.06	0.102	0.53	0.726	0.96
2	Eupatorin-5-methylether	0.005	0.01	0.473	0.35	0.982	0.88
3	Tiliroside	0.002	0.01	0.374	0.26	0.934	0.74
4	Cynarin	0.003	0.07	0.198	0.73	0.716	0.94
5	Baicalein	0.005	0.04	0.637	0.72	0.998	0.96
6	Hispidulin	0.040	0.07	0.661	0.54	0.976	0.87
7	13, II18-Biapigenin	0.005	0.01	0.582	0.07	0.985	0.64
8	Verbascoside	0.005	0.01	0.134	0.26	0.727	0.73
9	3,5-Dicaffeoylquinic acid	0.006	0.01	0.445	0.17	0.869	0.76
10	Puerarin	0.006	0.08	0.123	0.71	0.742	0.97
11	Glycitein	0.027	0.03	0.603	0.60	0.992	0.93
12	Kaempferol	0.011	0.01	0.692	0.66	0.974	0.90
13	Taxifolin	0.011	0.05	0.627	0.60	0.987	0.95
14	Eupatorin	0.060	0.00	0.598	0.05	0.977	0.49
15	Naringin	0.000	0.00	0.063	0.22	0.661	0.79
16	Apigenin-7-O-glucoside	0.005	0.01	0.195	0.31	0.802	0.82
17	Theophylline	0.000	0.02	0.306	0.31	0.884	0.77
18	Theobromine	0.000	0.01	0.176	0.39	0.781	0.86
19	Theaflavin	0.000	0.01	0.493	0.66	0.976	0.91
20	Quercetin	0.021	0.06	0.631	0.53	0.958	0.96

A reverse test was also performed. The query molecule defined by its R_F values in LPDS, MPDS and HPDS was compared to the database for a number of rows (four each) matching the specified similarity. To calculate the similarity, the Euclidean distance was selected and the four nearer neighbors (most similar) were displayed in an additional column.



4

Use of the database for proposal of potential matches

Conclusion

The examples above illustrate the potential of the CDS and its combination with machine learning. In this study, R_F values can be predicted, emphasizing that this feature is encoded within the chemical structure of the molecules. Moreover, the link between chemical structures and R_F allows to generate a list of four molecules likely to correspond to an unknown zone in complex mixtures. This prediction would be even more useful, if additional data such as mass and UV-VIS spectra were added to the database.

Further information is available on request from the author(s).

[1] T.K.T. Do, M. Schmid, I. Trettin, M. Hänni, E. Reich, Complementary developing solvents for simpler and more powerful routine analysis by high-performance thin-layer chromatography, JPC – J. Planar Chromatogr. – Mod. TLC. (2022). https://doi.org/10.1007/s00764-022-00185-1.

[2] T.K.T. Do, I. Trettin, M. Hänni, E. Reich, Applying machine learning to the data obtained with the complementary developing solvents protocol, accepted in J. Liq. Chromatogr. Relat. Technol. (2023).

[3] T.K.T. Do, M. Schmid, M. Phanse, A. Charegaonkar, H. Sprecher, M. Obkircher, E. Reich, Development of the first universal mixture for use in system suitability tests for High-Performance Thin Layer Chromatography, J. Chromatogr. A. 1638 (2021) 461830. https://doi.org/10.1016/j.chroma.2020.461830.

Contact: Dr. Tiên Do (tien.do@camag.com)

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HPTLC – A good technique for extractable and leachable studies of plastics

From left: Dr. Jigna Vadalia, Dr. Navin Sheth, Dr. Kashyap Thummar, Mr. Nitish Mandal

Dr. Kashyap Thummar, Assistant Professor at Graduate School of Pharmacy (GSP), Gujarat Technological University (GTU), India, employs chromatographic separation techniques, especially HPTLC, to develop new and improved quantitative analytical methods for determination of drugs, impurities, adulteration and naturally occurring compounds in a variety of sample matrices. He prefers HPTLC because it is flexible, inexpensive, time-saving and does not produce toxic waste.

Introduction

Phthalates are esters of phthalic acid that are commonly added to plastics to improve their flexibility, transparency, durability, and longevity. These phthalates are easily released into the environment from plastic and can harm all living organisms. Phthalates enter the body by contact to plastics, *e.g.* through ingestion, inhalation, skin absorption, and intravenous injection.

For the detection of extractable and leachable phthalates in pharmaceutical products, a simple, rapid, precise, and accurate HPTLC method was developed. It simultaneously estimates the presence of four different phthalates in various pharmaceutical products and containers: dimethyl phthalate (DMP), diethyl phthalate (DEP), dibutyl phthalate (DBP) and di(2-ethylhexyl) phthalate (DEHP). The method was successfully applied for determination of extractables and leachables from 12 parenteral products packed in plastic containers.

Standard solutions

10.0 mg of each phthalate were individually dissolved in 10.0 mL of methanol and subsequently combined to prepare a working standard solution at a concentration of 0.1 mg/mL of each.

Sample preparation

2.0 g of the plastic container (extractable) and 30.0 mL of the product packed in it (leachable) are extracted 3 times with 30.0 mL of *n*-hexane by 10 min of sonication. The organic layers are collected, evaporate to dryness and reconstituted with 1.0 mL of methanol.

Chromatogram layer

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

Sample application

Samples and standard solutions are applied as bands with the Linomat 5, 20 tracks, band length 5.0 mm, distance from left edge 10.0 mm, distance from lower edge 8.0 mm. 20.0 µL for sample solutions and 1.0–14.0 µL for standard solutions (8 points for calibration) are applied.

Chromatography

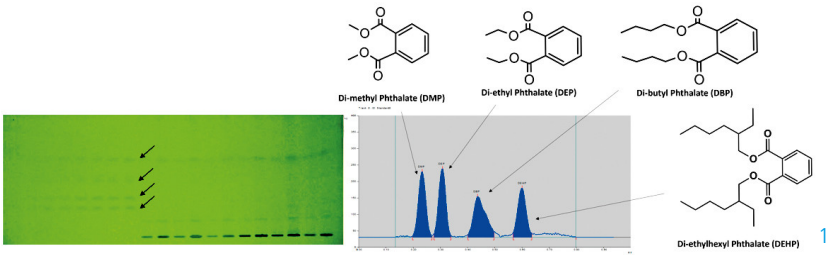
Plates are developed in a saturated Twin Trough Chamber (15 min, with filter paper) with *n*-hexane – ethyl acetate 9:1 (*V/V*) to a migration distance of 90 mm (from the lower edge), followed by drying with cool air for 5 min.

Documentation

Images of the plate are captured with the TLC Visualizer in UV 254 nm.

Densitometry

Absorbance measurement at 240 nm is performed with the TLC Scanner 4 and *winCATS*, slit dimension 4.00 mm × 0.30 mm, scanning speed 20 mm/s.

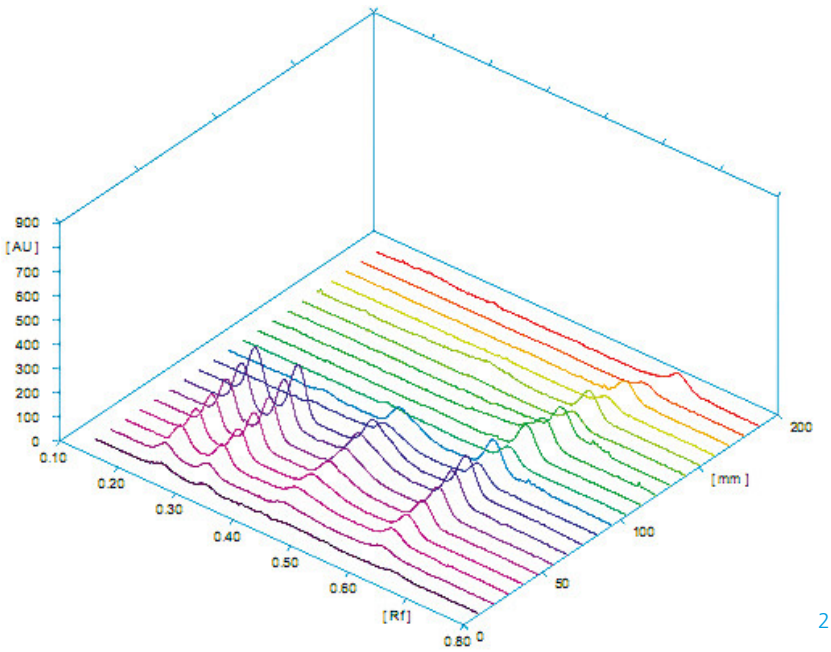


1

HPTLC chromatogram in UV 254 nm (left) and densitogram measured at 240 nm (right)

Results and discussion

A representative densitogram of samples is shown. During evaluation of the chromatogram, phthalates in simulated and test samples give the same R_F values as the standard and are well separated from matrix components. A simulated sample is a laboratory-made sample that is designed to mimic a real-world product or material.



2

3D profile of scanned sample and standard tracks (measured at 240 nm)

DMP, DEP, DBP and DEHP are separated at R_F values of 0.23, 0.31, 0.44 and 0.60 respectively. The limit of quantification was in the range of 41.7 to 99.8 ng/band for the four phthalates and linearity was established between 100.0 and 1400.0 ng/band. The presence of individual phthalates was found in 12 pharmaceutical products (all parenteral formulations) in significant amounts. Importantly, of the four phthalates, DEHP was found in all tested samples as an extractable and leachable.

The HPTLC method for detection of phthalates is cost-effective as compared to available analytical methods such as HPLC, LC-MS/MS, GCMS/MS etc. as these techniques require more solvent consumption, power consumption, analysis time, and involve complex sample preparation methods. The method can be universally applied to other samples apart from pharmaceutical products like water, food items *etc*. which are sold in plastic containers.

[1] T. H. Broschard *et al.* (2016) Regulatory Toxicology and Pharmacology 81, 201–211.

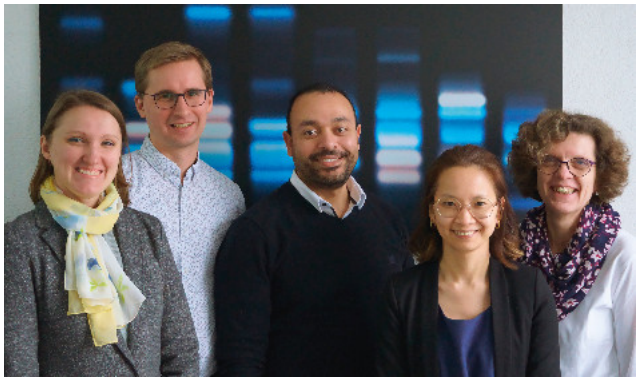
[2] K. Thummar and N. Sheth, Publication date: 2022/6/7, Patent office: IN, Patent number: 398670.

[3] K. Thummar *et al.* (2020) Analytical Chemistry Letters 10 (1), 93–103.

Further information is available on request from the authors.

Contact: Dr. Kashyap Thummar, Assistant Professor, Graduate School of Pharmacy, Gujarat Technological University, Gandhinagar, Gujarat, India, ap_kashya@gtu.edu.in

The new team at CAMAG laboratory



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

Dr. Tiên Do joined CAMAG in July 2014 as scientific support specialist, she became Deputy Head of Laboratory in 2017. Throughout her career at CAMAG, she has provided trainings and seminars, and developed HPTLC methods in various application fields. Customers worldwide appreciate her outgoing personality, generalist perspective, and ability to think out of the box when approaching an analytical problem. Since 2022, she is Head of Laboratory and leads the scientific research, maintains the overview on all projects of the lab, and assures generation of high-quality results for peer-reviewed publications. Her focus is on implementing applications of the new HPTLC PRO technology. She is striving to generate a scientific basis for emerging HPTLC concepts and practical applications that benefit industry and that meet commercial market needs.

Dr. Do is supported by a mostly new team of four experienced scientists:

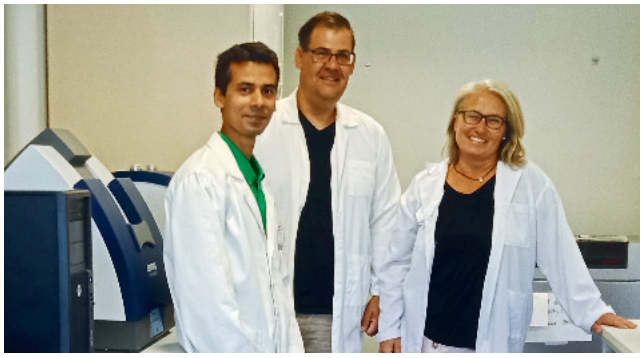
Ilona Trettin joined CAMAG in October 2014. She holds a German Diploma as Chemical and Pharmaceutical Technician. She has a long expertise in planar chromatography based on more than 30 years of work in a strictly regulated environment. In 2022, Mrs. Trettin became Laboratory Coordinator. In this position, she is responsible for supporting laboratory examinations and experiments, collecting laboratory samples, and ensuring the efficiency and performance of laboratory tools and equipment. As a scientist, Mrs. Trettin remains responsible for method development in collaborative projects with the European and the US Pharmacopoeia.

Dr. Kateryna Khokhlova joined CAMAG in April 2022 as a scientist. She is working on various projects in different application fields to support business development. In her previous career, she was an Associate Professor at Kharkiv National University of Pharmacy (Ukraine), where her research was focused on the use of HPTLC for the standardization of plant raw materials and herbal medicines of Ukrainian origin. After training at the USP (Rockville) in 2011, she participated in the development of national monographs on herbal drugs for the Ukrainian Pharmacopeia. Since 2019, Dr. Khlokhlova is the President and Secretary General of the Newly Independent States Chapter of the HPTLC Association.

Dr. Ehab Mahran is working as a scientist at CAMAG since June 2022. He obtained his MSc in Pharmaceutical Sciences at the University of Cairo (Egypt). Then, he obtained his PhD in Natural Products Chemistry from the Institute of Pharmacy at the University of Marburg, Germany, where he also pursued postdoctoral studies. Prior to joining CAMAG, Dr. Mahran held positions in academia and industry in various countries where he was involved in projects focused on natural product chemistry and bio-activity. At CAMAG, Dr. Mahran is responsible for method development in collaborative projects with the US Pharmacopoeia. He contributes to the development of new instrumentation and software and supports the development of the market.

Dr. Stefan Weiss joined CAMAG as a scientist in September 2022. He graduated in chemistry from the University of Applied Sciences in Aalen (Germany). Between 2008 and 2020, he worked in the laboratory of the long-distance water supplier ZV Landeswasserversorgung in Langenau in Southern Germany. He was working on effect-directed analysis in combination with HPTLC. Then, he worked in the field of quality assurance at Carbogen AMCIS (Switzerland), a manufacturer of active pharmaceutical ingredients. At CAMAG, Dr. Weiss contributes to the development of new HPTLC PRO instruments and software and is responsible for method development of new application fields in HPTLC PRO.

Application of an HPTLC method for detection and quantification of 5-hydroxymethylfurfural in honey



Left to right: MD Khairul Islam, Tomislav Sostaric, Dr. Cornelia Locher

The research team at the University of Western Australia (UWA), Division of Pharmacy, developed an HPTLC based real-time honey assessment tool for beekeepers and packers to determine a honey's floral source alongside the collation of key phytochemical parameters and bioactivity data for a wide range of Australian honeys. Currently, the team is using HPTLC as a qualitative and quantitative honey analysis tool. They monitor changes over time, and caused by storage and handling conditions.

Introduction

Honey is a sweet natural product appreciated for its unique flavor, color and bioactivity. Honey is produced by honeybees from flower nectar. Raw honey contains phenolics, flavonoids, proteins, vitamins and minerals, however, it is rarely sold on the market in the raw form. Before being bottled and packaged, honey undergoes several processing steps, including filtration, radiation and/or heating. Excessive or prolonged heating can have detrimental effects to the honey's quality. It is known to produce potentially toxic chemicals like the Maillard reaction product 5-hydroxymethyl-furfural (HMF), which is suspected to have carcinogenic effects when ingested in high doses. An HPTLC based method can be used for the fast and cost-effective assessment of the HMF content of honey and thus presents a convenient honey quality control tool.

The applied method is rapid, reliable, and repeatable and, therefore, a convenient analytical tool for routine quality control of honey. The method involves a simple dissolution step followed by a short chromatographic development time (9–10 min) without chamber saturation or derivatization. Up to 10 samples can be analyzed on a single plate with only small sample quantities (approx. 1 g) required.

Standard solution

Aqueous 0.008% (w/v) freshly prepared HMF solution.

Sample preparation

An artificial (ART) honey is prepared by dissolving 40.5 g fructose, 33.5 g glucose, 1.5 g sucrose and 7.5 g maltose in 17 mL of deionized water. The ART is individually kept at 40 °C, 60 °C and 80 °C. Sampling is done at time of preparation (t_0), 6 h, 12 h, 24 h, 48 h and then over a period of four months. For HPTLC analysis, the collected honey samples are prepared as 1 g/10 mL aqueous solutions.

Chromatogram layer

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

Sample application

Samples and standard solutions are applied as bands with Linomat 5, 15 tracks, band length 8.0 mm, distance from left edge 20.0 mm, distance from lower edge 8.0 mm.

Chromatography

Plates are developed in the ADC 2 without chamber saturation with ethyl acetate as mobile phase to the migration distance of 50 mm (from the lower edge), followed by drying for 5 min.

Documentation

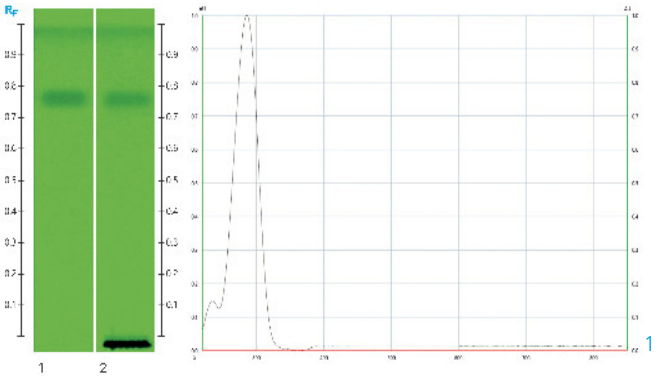
Images of the plate are captured with the TLC Visualizer 2 in UV 254 nm.

Densitometry

To find out the absorption maximum for HMF, a spectral scan is performed using the TLC Scanner 4 from 220 nm – 850 nm both on the bands of pure HMF and HMF bands produced in artificial honey treated at elevated temperature. Based on these scans, HMF analysis in honey samples is carried out at 290 nm using the TLC Scanner 4.

Results and discussion

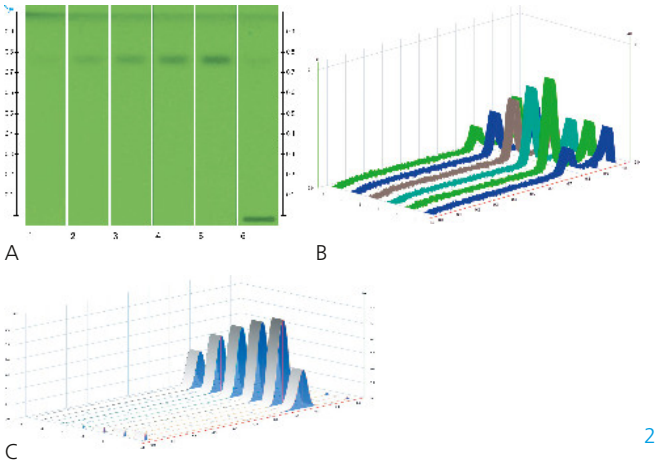
The following figure shows the HPTLC fingerprints of pure HMF and HMF produced during storage at elevated temperature. During the analysis, the HMF is completely separated from the honey matrix, and both pure and newly produced HMF appear at R_F 0.76. Positive identity of HMF is indicated by spectra comparison of standard and sample. The absorbance maximum is identified at 290 nm.



1

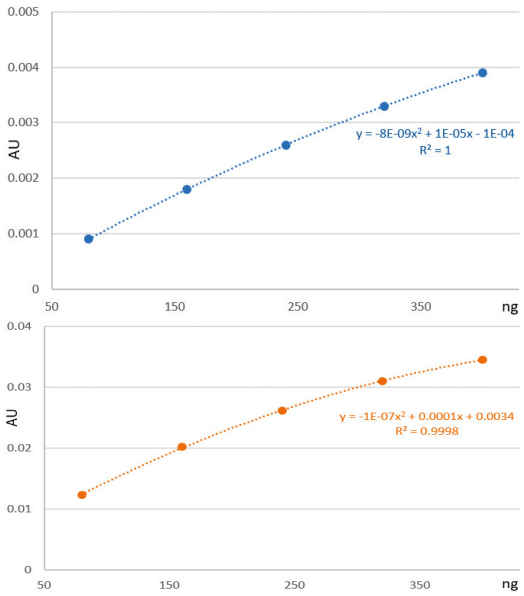
HPTLC image of HMF in UV 254 nm (left; track 1: Standard HMF and track 2: HMF produced in honey stored at elevated temperature) and UV-VIS spectra of HMF from 220 – 850 nm (right).

For quantification and to prepare the HMF standard curve, 1.0, 2.0, 3.0, 4.0 and 5.0 µL of the respective standard solution are applied. For the analysis of HMF in the honey samples, 10.0 µL of the respective honey solution is applied at a rate of 30.0 nL/s. After development, Peak Profiles from Images (PPI) obtained in UV 254 nm with the TLC Visualizer 2 are compared with Peak Profiles from Scanning Densitometry (PPSD) subsequently measured at 290 nm with the TLC Scanner 4.



2

(A) HPTLC images in UV 254 nm; (B) Peak Profiles from Images (PPI), UV 254 nm with TLC Visualizer 2; (C) Peak Profiles from Scanning Densitometry (PPSD), 290 nm with TLC Scanner 4; (track 1–5: standard tracks and track 6: 10.0 µL honey sample solution)

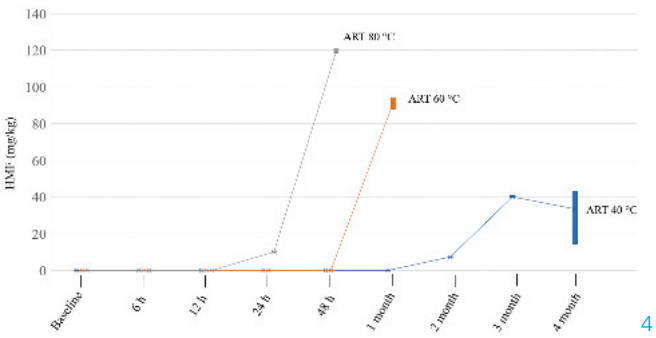


3

Standard curve prepared using the data from PPI UV 254 nm (top) and at PPSD UV 290 nm (bottom)

Editor's note: The response of HMF is higher for scanning densitometry compared to image-based evaluation; the working range can be adjusted to the linear working range by reducing the concentration of the sample and standard solutions.

The level of HMF in artificial honey was within the acceptable limit (80 mg/kg of honey) after 4 months of storage at 40 °C. The HMF limit exceeds the acceptable limit after 48 h for artificial honey stored at 60 °C. For the honey stored at 80 °C, the limit is exceeded already after 24 h of storage. This experiment shows that honeys need to be stored or temperature treated carefully to limit the formation of HMF.



4

HMF content in artificial honey stored at different temperatures over time

Conclusion

The HPTLC method for the detection and quantification of HMF in honey is easy to perform and offers a convenient quality control tool for the honeybee industry. It allows monitoring the HMF-related changes to the quality of honey during processing (especially temperature treatment) and storage. The absence of any sample pre-treatment steps and post-chromatographic derivatization, a neat solvent as developing solvent, and no chamber saturation and activation are major advantages. The method may also be used for the detection and quantification of HMF in other botanicals and foods with high sugar content.

[1] M. K. Islam *et al.* Foods (2021), 10(2), 357.
[2] M. K. Islam *et al.* Molecules (2022), 27(23), 8491.
[3] M. K. Islam *et al.* Molecules (2020) 25(22).
[4] E. S. Chernetsova, Anal Bioanal Chem (2011), 401(1), 325-332.

Further information is available on request from the authors.

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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 × 10 cm)
- Software-controlled by *visionCATS*

Further information:
www.camag.com/PROderivatization

HPTLC for stability testing of dihydroartemisinin

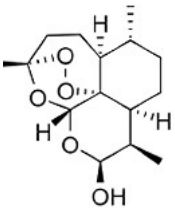
From left: Akshay Charegaonkar, Tukaram Thite, Amit Palande, Dr. Saikat Mallick

Amit Palande, application specialist under the guidance of Akshay Charegaonkar (managing director), Tukaram Thite (senior lab manager) and Dr. Saikat Mallick (lab manager) work at Anchrom Enterprises Pvt Ltd, Mumbai, India. The company specializes in instrumental planar chromatography, and develops new, quantitative, and regulatory compliant analytical methods for pharmaceutical formulations, APIs, herbal products, food products, organic intermediates, dyes etc. Mr. Palande benefits from HPTLC because it is a fast, simple, economical and flexible, "visible chromatography" technique. HPTLC is risk-free and multiple detections can be made without repeating chromatography. This is especially helpful for dihydroartemisinin, because it is not UV absorbing and needs derivatization for densitometric detection.

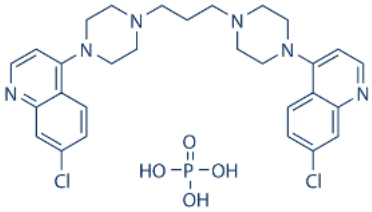
Introduction

Dihydroartemisinin (also known as dihydroqinghaosu, artenimol or DHA) is a drug used to treat malaria. It is globally recognized for its efficacy and safety in the clinical treatment of malaria for decades. DHA is the active metabolite of all artemisinin compounds (artemisinin, artesunate, artemether, etc.) and is also available as a drug by itself. It is a semi-synthetic derivative of artemisinin and is widely used as an intermediate in the preparation of other artemisinin derived antimalarial drugs. DHA is often combined with piperaquine phosphate (PPQ). Like any formulation, these tablets need to be tested for shelf life *i.e.* stability.

Shelf-life studies are performed by accelerated or forced degradation studies as per ICH guidelines Q 1 A (R2).



Dihydroartemisinin



Piperaquine phosphate

HPTLC is well suited for stability studies, because it is inexpensive and time-saving. Accelerated degradation studies need a very large number of samples to be analyzed. By HPTLC,15–20 samples can be quantified simultaneously on one plate in about 40–80 minutes. The solvent consumption is only about 20 mL for those 20 samples and little waste is produced. It was separately established, that piperaquine phosphate did not degrade in the studies. Hence a method was developed that kept piperaquine phosphate at the base of the chromatogram but selectively moved DHA and its two degradation products. DHA and the degradation products were detected by simple derivatization and then quantitatively evaluated.

HPTLC is a cost-effective and time-saving technique for the pharma industry, which deals with a heavy load of samples, also competition to introduce a new formulation is very intense. The presented method is a green method, which only uses 20 mL of solvent for 15–20 samples and produces almost no waste. Since the degradation products are unknown, the common procedure of establishing the calibration function using the diluted main substance (DHA in this case) was applied. Many times, as in this case, the samples have to be overloaded to detect the small quantities of impurities/degradation products.

Standard solutions

A stock solution of 1.0 mg/mL of dihydroartemisinin in acetonitrile is prepared and diluted 1:20 (*V/V*) for analysis.

Sample preparation

Ten tablets containing 40.0 mg of dihydroartemisinin and 320.0 mg of piperaquine phosphate are milled. Powdered tablets equivalent to 100.0 mg of DHA are transferred in a 20.0 mL volumetric flask and dissolved in acetonitrile. The supernatant after centrifugation is used for application.

Chromatogram layer

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

Sample application

Samples and standard solutions 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. 10 µL for sample and standard solutions are applied.

Chromatography

Plates are developed in a 20 x 10 cm Twin Trough Chamber with chamber saturation (with filter paper) for 20 min, development with cyclohexane – ethyl acetate – glacial acetic acid 10:5:1 (*V/V*) to the migration distance of 90 mm (from the lower edge), followed by drying for 5 min.

Post-chromatographic derivatization

Plates are sprayed with 3.0 mL of anisaldehyde-sulfuric acid reagent (to 170 mL of cooled methanol, 20 mL of acetic acid and 10 mL of sulfuric acid are added, after cooling to room temperature 1 mL of anisaldehyde is added) using the CAMAG Derivatizer. After spraying, the plates are heated at 110 °C for 5 min using the TLC Plate Heater.

Documentation

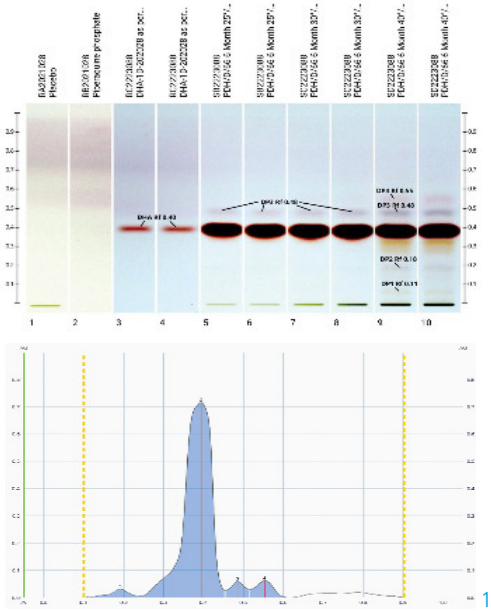
Images of the plate are captured with the TLC Visualizer in UV 366 nm and white light.

Densitometry

Absorbance measurement at 540 nm (tungsten lamp) is performed with CAMAG TLC Scanner 4 and *visionCATS*, slit dimension 6.00 mm x 0.45 mm, scanning speed 20 mm/s, evaluation via peak area.

Results and discussion

The determination of the degradation product with the mobile phase cyclohexane – ethyl acetate – glacial acetic acid 10:5:1 (*V/V*) is verified by the positions of the individual drugs, where PPQ remains at the application position and DHA moves to hR_F 40. Any other zones at different hR_F are reported as degradation products. The placebo shows no zones in the chromatogram. Prior to derivatization, no zones are visible. Quantitative evaluation is performed after derivatization by densitometric absorbance measurement at 540 nm. The data is recorded and used to calculate the degradation products. Total impurities in the samples are found to be within the 1.5% limit as per specification, defined in house. A representative densitogram of a stability batch sample and standard is shown. The area of each impurity is calculated as follows and then summarized.



1

Top: image in white light after derivatization: Track 1: placebo, track 2: piperaquine phosphate, tracks 3, 4: DHA standard, tracks 5–10: different formulations of tablets in the stability study (DP = degradation product); bottom: densitogram at UV 540 nm of a tablet batch in which degradation products were detected.

Results of a 6 months old batch

T in °C / rH in %	hR_F	Sum of unknown impurities
25 °C / 60 %	48	0.13 %
30 °C / 65 %	48	0.13 %
40 °C / 75 %	11	0.03 %
	18	0.12 %
	48	0.15 %
	55	0.21 %

[1] CAMAG Application Note A-86.1: Determination of artemisinin in *Artemisia annua* leaf by HPTLC

Further information is available on request from the authors.

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HPTLC-based fingerprinting of agave fructooligosaccharides

Mercedes G. López and Luis F. Salomé (left), Patricia A. Santiago (top) and Ruth E. Márquez (bottom)

Mercedes G. López and Luis F. Salomé work on agave fructooligosaccharides (aFOS, fructans) at the Research and Advanced Studies Center (CINVESTAV)-IPN in Mexico. Patricia A. Santiago and Ruth E. Márquez also conduct fructan research at the Interdisciplinary Research Center for the Integral Regional Development (CIIDIR)-IPN in Mexico. The group focuses on fructan chemical characterization and their biological effects as prebiotics on the human health.

Introduction

Fructans are a polydisperse mixture of fructose polymers, and contain only one or no glucose in their structures. They are commonly found in agaves and possess several industrial applications. Fructans have been mainly used as prebiotics and food supplements. Moreover, they are directly correlated with the yield and quality of the alcoholic drink tequila. The standard techniques for the characterization of fructans is high performance anion exchange chromatography (HPAEC), which is time consuming (up to 80 min/sample). The HPTLC technique allows the parallel analysis of many samples with little solvent consumption.

Thus, this study aimed at exploring the potential of the aFOS fraction as a good descriptor of the fructan differentiation in agave species through age, and at the feasibility of HPTLC as a robust fingerprinting platform through multivariate data analysis (MVDA). HPAEC was used for comparison with HPTLC.

The proposed method is rapid, accurate and precise. It is suited as a high-throughput method with a significant reduction in working time, supplies and solvents. Finally, it produces robust data which can be used for multivariate modelling.

Standard solutions

2.0 mg of glucose, fructose, sucrose, 1-kestose, 1-nystose, and 1-F fructofuranosylnystose (DP5) are dissolved in ethanol – water 7:3 (*V/V*).

Sample preparation

Agave fibers (*Agave potatorum* and *Agave angustifolia*) are extracted in aqueous ethanol (80%) for 1 h at 60 °C, reextracted twice with pure water, and then defatted with chloroform. The aqueous phase is reduced and spray-dried. Samples are prepared at 7.0 mg/mL. They are firstly dissolved in 0.3 mL of water and then filled to 1.0 mL with absolute ethanol [1].

Chromatogram layer

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

Sample application

Samples and standard solutions are applied as bands with the Automatic TLC Sampler (ATS 4), 17 tracks, band length 6.0 mm, distance from left edge 20.0 mm, distance from lower edge 10.0 mm and 10.0 mm between bands. 5.0 µL for sample solutions and 1.0 µL for standard solutions are applied.

Chromatography

Plates are developed in the ADC 2 with chamber saturation (with filter paper) for 20 min and after activation at 47 % relative humidity for 10 min using a saturated solution of potassium thiocyanate. The first development is performed with isopropanol – butanol – water – acetic acid 14:10:4:2 (*V/V*) to the migration distance of 75 mm (from the lower edge), followed by drying for 5 min. The second development is performed with iso-propanol – butanol – water – formic acid – acetic acid 14:10:4:1:1 (*V/V*) to the migration distance of 85 mm (from the lower edge), followed by drying for 5 min.

Post-chromatographic derivatization

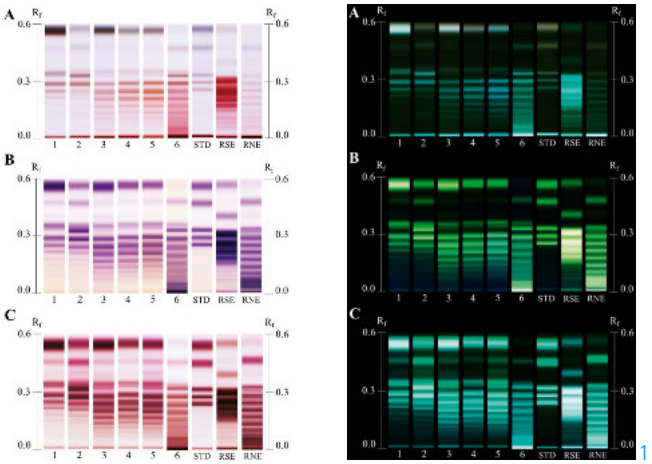
The plates are immersed into a solution of diphenylamine-aniline-phosphoric acid (referred to as aniline) using the Chromatogram Immersion Device, immersion speed 2 cm/s and immersion time 2 s, dried for 30 s with cold air and heated at 120 °C for 3 min using the TLC Plate Heater. The same samples are also derivatized with α-naphthol and orcinol at 110 °C [1].

Documentation

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

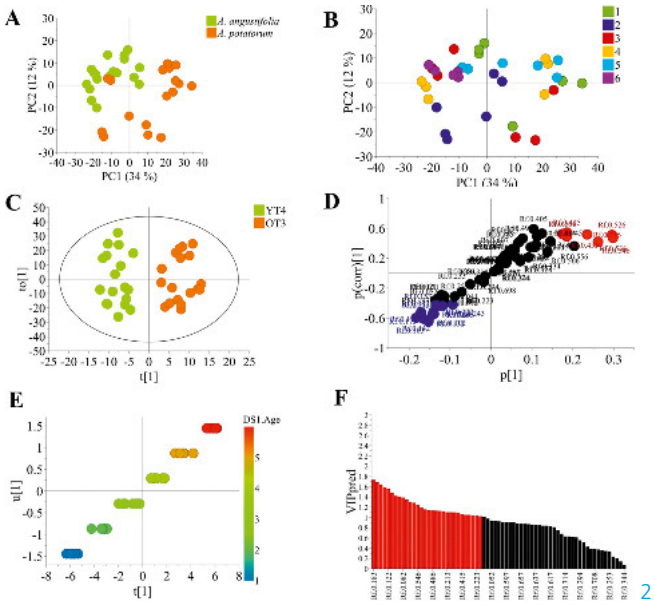
Results and discussion

The information produced by HPAEC was able to differentiate agave specimens according to their species and age. Moreover, the data was also good for creating supervised models. The HPAEC models indicated a decrease of simpler sugars such as fructose, glucose and sucrose, while fructans with higher degree of polymerization (DP) are synthetized as the agave age increases. The visual inspection of HPTLC chromatograms, independent of the derivatization reagent, showed the same trend. Also, it was observed that α-naphthol and orcinol produced more intense monochromatic bands, while aniline produced bicolor patterns.



1 *(Left) Processed HPTLC chromatograms in white light (according to [1]) of representative Agave potatorum samples and (right) negative-HPTLC chromatograms, after derivatization with **A** aniline, **B** α-naphthol and **C** orcinol. STD, standard mixture; RSE, raftilose; RNE, raftiline. Track number indicates agave age expressed in years. Reproduced from [1]. (https://creativecommons.org/licenses/by/4.0/legalcode).*

For MVDA, the intensity values of the peak profiles from images (PPI) were inverted during data extraction [1] and negative-HPTLC chromatograms were processed with an open-source-software in all color channels according to [2]. The data was normalized to the quality control sample track in each corresponding plate. Furthermore, the data was scrutinized by principal component analysis (PCA), orthogonal projection to latent structures discriminant analysis (OPLS-DA) and orthogonal projection to latent structures (OPLS) analysis. Subsequently, data was approached by MVDA and a PCA showed a clear separation dictated by species. Thus, we concluded that the aFOS fraction was enough to describe agavins metabolism and that HPTLC data was robust enough to be combined with MVDA, which produced even better supervised models than HPAEC.



2 *MVDA of HPTLC data approached by **A** PCA colored according to species; **B** PCA colored according to age; **C** OPLS-DA **D** S-plot of OPLS-DA; **E** orthogonal projection to latent structures for carbohydrate/age correlation; **F** VIP_{pred}-plot. Reproduced from [1]. (https://creativecommons.org/licenses/by/4.0/legalcode).*

An extended version of this article can be assessed at https://bit.ly/41gdcnq.

[1] L.F. Salomé-Abarca, *et al.* (2023) Curr. Res. Food Sci. 100451. doi: 10.1016/j.crfs.2023.100451.

[2] D. Fichou, *et al.* (2016) Anal. Chem. 12494. doi: 10.1021/acs.analchem.6b04017.

Further information is available on request from the authors.

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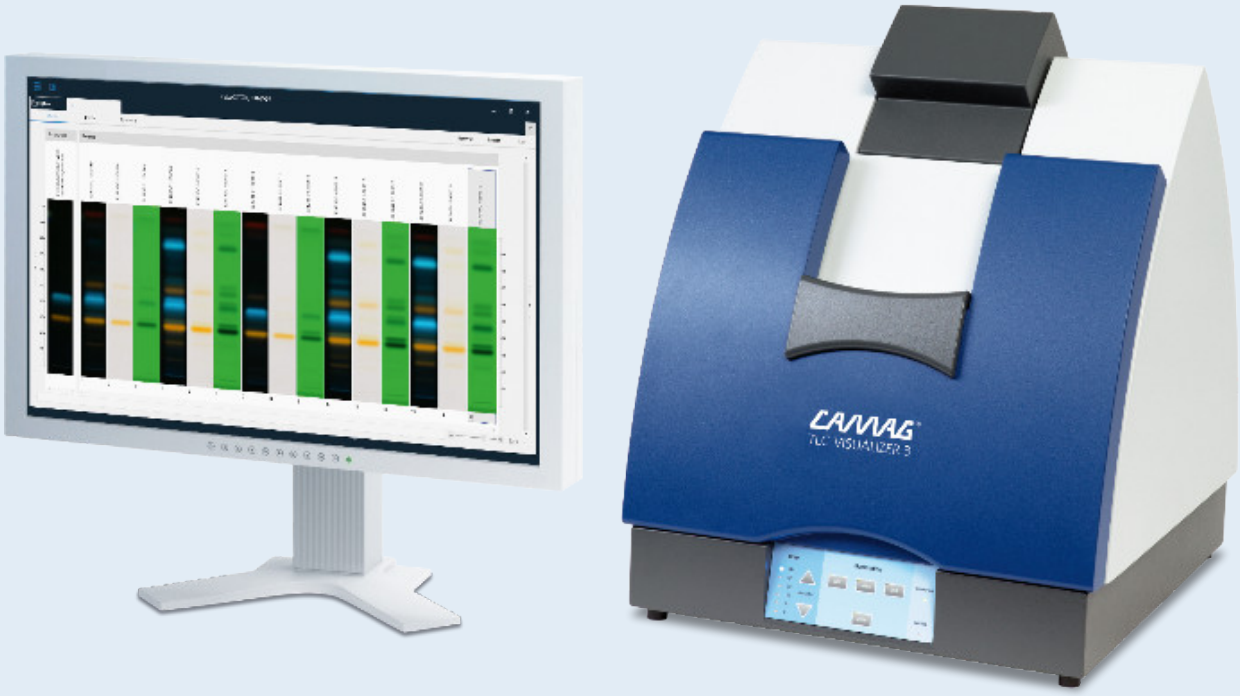
CAMAG® TLC Visualizer 3



High-end imaging and documentation system for white light and UV fluorescence detection

The TLC Visualizer 3 is a high-end imaging and documentation system with superior performance in white light, long-wave UV (366 nm) and short-wave UV (254 nm) ensuring illumination with maximum homogeneity.

Designed for the acquisition of premium-quality images, the documentation system features a high-performance industrial camera of the newest generation equipped with a CMOS image sensor.

Powered by *visionCATS* HPTLC software, the TLC Visualizer 3 allows the detection of even the faintest zones. Designed for supporting the HPTLC workflow, the best-in-class HPTLC software features a variety of sophisticated image enhancement tools exploiting the full potential of the TLC Visualizer 3 and enables to display references and samples originating from the same and/or different plates in various illumination modes side by side.

The high-end device ensures reproducibility at a maximum level, while maintaining cGMP/GLP and 21 CFR Part 11 compliance.

Further information:
www.camag.com/tlcvisualizer3

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