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CAMAG (Switzerland)
Sonnenmattstrasse 11 • 4132 Muttenz
Phone +41 61 4673434 • Fax +41 61 4610702
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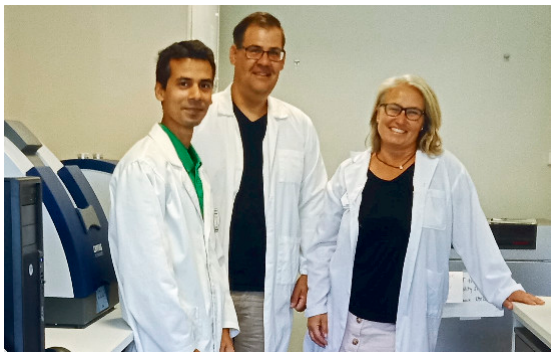
Analysis of honey by HPTLC



From left: Md Khairul Islam, Tomislav Sostaric, Dr. Cornelia Locher

The research team at the University of Western Australia (UWA), Division of Pharmacy, in collaboration with the Cooperative Research Centre for Honey Bee Products Limited (CRC-HBP) develops a 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. The team currently also investigates potential correlations between phytochemical characteristics of honeys and their bioactivity. Using HPTLC as a qualitative and quantitative honey analysis tool, they monitor changes over time, and caused by storage and handling conditions. Moreover, a HPTLC based method for the detection of sugar syrup adulterants in honey has also recently been developed.

Introduction

Honey is derived from nectar collected by honey bees from a range of floral sources. After collection, numerous processes take place outside and also within the hive, such as exposure to bee related enzymes and removal of moisture, which ultimately convert the nectar into honey. Honey can be considered a complex natural product, consisting of a high amount of sugars (>70%), residual moisture (typically 17–20%) and a small portion (approx. 3%) of non-sugar constituents, including minerals, vitamins, protein, phenolics and flavonoids. These minor constituents are directly related to the honey's nectar source and thus play a critical role in the authentication of honeys. They also influence the honey's organoleptic and physicochemical characteristics as well as its level of bioactivity. Antioxidant activity is linked to high concentrations of phenolics, phenolic acids, anthocyanins, hydroxycinnamic acid derivatives and flavonoids. DPPH* (2,2-diphenyl-1-picrylhydrazyl, $C_{18}H_{12}N_5O_6$) is a powerful radical used for the antioxidant screening of compounds, mixtures of constituents, extracts and biological matrices, such as wine, fruit juices, salivary secretions and plant extracts, and also, as demonstrated in this study, honey.
The phytochemical composition of honeys depends on the floral nectar source and thus on the geographic origin and time of harvesting. It

might also be influenced by processing and storage conditions, by the bee species collecting the nectar and the bee enzymes the nectar and honey come in contact with. Despite this complexity, honeys from the same floral species present characteristic constituent patterns, which can be used in the authentication of their floral source. These patterns can be effectively captured by HPTLC analysis of honeys' organic extracts and can thus assist in the authentication of their floral origins.

As a major source of energy, carbohydrates, including simple sugars such as glucose, fructose, sucrose, maltose or galactose, play an important role in human nutrition. The determination of their content in various botanical products and food items is therefore of interest. However, the quantification of simple sugars is not without challenges due to their high polarity, low volatility and lack of a sizeable chromophore. Furthermore, these sugars are often found in complex matrices, which require their separation from proteins, fats, and / or minerals as well as other matrix constituents prior to analysis.

Herein, three different methods for the analysis of honey samples are presented. Method **A** is suited for the honey floral source identification by HPTLC fingerprinting, method **B** enables a fast assessment of antioxidant zone activity, and method **C** allows to quantify sugars and to detect sugar adulterants in honey.

The developed authentication method (A) is rapid, reliable, and repeatable and can therefore be used as a convenient analytical tool in routine honey quality control. The method involves a simple solvent extraction step followed by a short chromatographic development time (9 –10 min). It requires minimal solvent and reagent input while allowing the simultaneous analysis of up to 14 samples on a single plate. HPTLC-DPPH analysis (B) is advantageous over traditional assays which measure the total antioxidant activity, as it can detect individual active compounds and quantify their respective contribution to the overall antioxidant effect. The analytical approach is easy and fast to perform and powerful in visualizing antioxidant compounds. Further, their respective activity can be quantified as gallic acid equivalents, even if their chemical identity is unknown. The developed method for sugar analysis (C) is convenient and easy to perform in a single development step with minimal sample pre-treatment. Moreover, only small sample quantities (approx. 100 mg) and small volumes of development solvent (approx. 35 mL) are needed for the analysis of multiple samples in a single run, which makes the method also a very cost effective approach to easily detect and quantify major sugars in honey.

Standard solutions

(A) Reference solution of 4,5,7-trihydroxy-flavanone (0.5 mg/mL) is prepared in methanol.

(B) Standard stock solution of gallic acid (20 µg/mL) is prepared in methanol.

(C) Standard glucose, fructose, sucrose and maltose solutions (250 µg/mL) are prepared in 50% aqueous methanol with sonication.

Sample preparation

(A and B) Manuka, Jarrah, Banksia and Marri honeys, and a honey of an unidentified floral source referred here as UNF (Supermarket honey; unidentified floral origin); 1 g of each honey is mixed with 2 mL of deionized water, then extracted three times with 5 mL of dichloromethane. The combined organic extracts are dried at ambient temperature and reconstituted in 100 µL of dichloromethane prior to analysis.

(C) Two honeys (Manuka and Jarrah); four different sugar syrups (Maple, Corn, Golden, and Glucose); and artificially adulterated Manuka and Jarrah honey with four different sugar syrups at 30% concentration. The sample solutions (1 mg/mL) are prepared using 50% aqueous methanol.

Chromatogram layer

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

Sample application

With the Linomat 5 between 1.0 –7.0 µL of the standard and 3.0 –5.0 µL of each sample solution are applied as 8 mm bands at 8 mm from the lower and 20 mm from the left edge of the HPTLC plate (dosage speed 150 nL/s for A and B and 50 nL/s for C).

Chromatography

(A and B) With the Automatic Development Chamber (ADC 2) the plates are conditioned to 33% relative humidity, pre-conditioned for 5 min, and developed to a distance of 70 mm in a saturated chamber (20 min for saturation). The developing solvent is toluene – ethyl acetate – formic acid 6:5:1 (*v/v*) [1, 2, 3]. Plates are dried for 5 min in the ADC 2.

(C) With the Automatic Development Chamber (ADC 2) the plates are conditioned to 33% relative humidity, pre-conditioned for 5 min, and developed to a distance of 85 mm in a saturated chamber (60 min for saturation). The developing solvent is 1-butanol – 2-propanol – aqueous boric acid (5 mg/mL) 30:50:10 *(v/v/v)* [5]. Plates are dried for 5 min in the ADC 2.

Post-chromatographic derivatization

(A) The plates are derivatized with 3.0 mL of vanillin reagent using the Derivatizer (yellow nozzle, level 3) and heated for 3 min at 115 °C using the TLC Plate Heater.

(B) The plates are derivatized with 2.0 mL of 0.4% DPPH reagent using the Derivatizer (yellow nozzle, level 1).

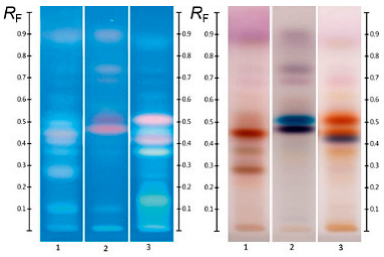
(C) The plates are derivatized with 2.0 mL of aniline – diphenylamine – phosphoric acid reagent using the Derivatizer (yellow nozzle, level 5). The derivatized plates are heated for 10 min at 115 °C using the TLC Plate Heater.

Documentation

With the TLC Visualizer 2 at UV 254 nm and UV 366 nm after development (A) and in white light (A, B*, C) and UV 366 nm (A) after derivatization (for B 60 min after reagent transfer).

Results and discussion

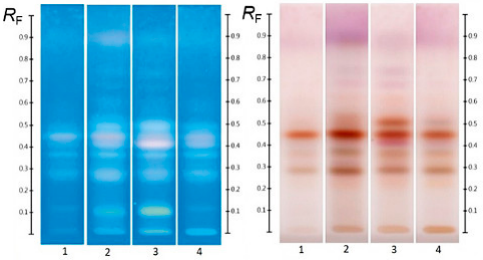
(A) The following figure shows the HPTLC fingerprints of three different honeys, labelled as Jarrah, Banksia and Marri, obtained at UV 366 nm and white light after derivatization. The two sets of images show a unique pattern for each honey within a R_F range of 0.05 to 0.60, representative of each honey's specific fingerprint.



1

Different honeys labelled as "Jarrah" (track 1), "Banksia" (track 2) and "Marri" (track 3); image at UV 366 nm (left) and white light (right) after derivatization with vanillin reagent

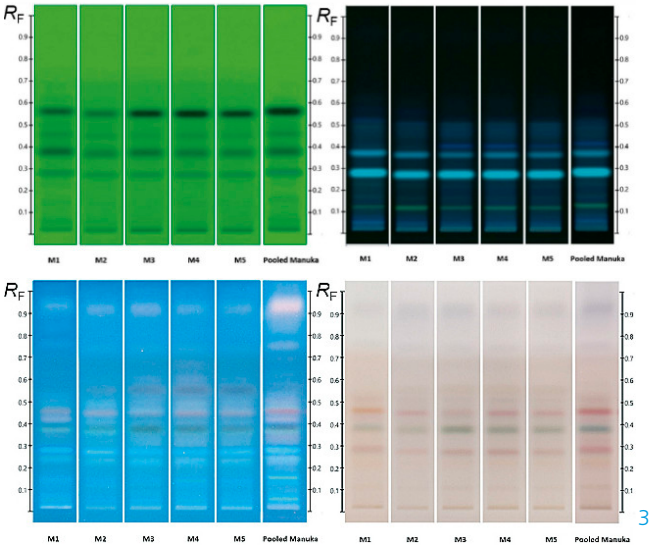
Four different honeys, all purchased labelled as Jarrah honeys, were analyzed at UV 366 nm and at white light after derivatization. Though obtained from different suppliers, all honeys share common zones between R_F 0.05 to 0.60 in their fingerprint, which can serve as a convenient authentication tool.



2

Different honeys labelled as "Jarrah"; image at UV 366 nm and white light after derivatization with vanillin reagent

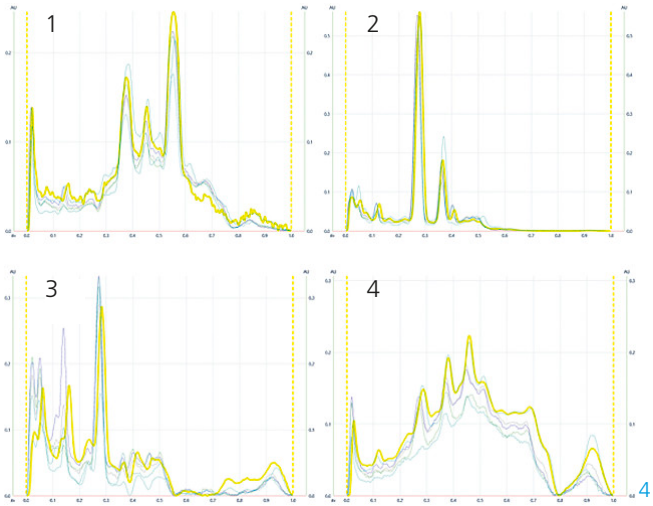
As a natural product, the composition of honey, even when derived from the same floral nectar, can be expected to exhibit some natural variation between samples. In order to obtain a representative fingerprint and to limit the impact of natural variation on the effectiveness of the method as an authentication tool, individual samples were pooled to produce an 'average' fingerprint, in which major deviations from the norm were 'diluted out' and key compounds, representative of the floral source across all samples, were amplified.



3

HPTLC fingerprints of several Manuka samples along with pooled sample; (from left to right) images at UV 254 nm, UV 366 nm after development and UV 366 nm and white light after derivatization

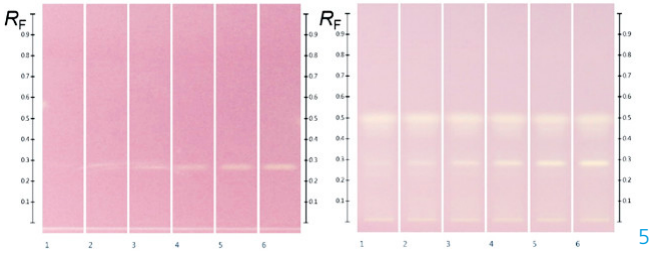
The effectiveness of this approach is demonstrated in the figure above where a pooled HPTLC fingerprint captures the key features of five individual samples, all labelled as Manuka honey. Slight variations present in the Manuka samples (*e.g.* zones at R_F 0.41 at UV 366 nm after development and at R_F 0.55 and R_F 0.38 at UV 366 nm after derivatization, as well as individual zone intensities, which correspond to compound concentrations) are no longer evident in the pooled fingerprint.



4

HPTLC peak profiles from images (PPI) of Manuka samples along with pooled sample highlighted in yellow at UV 254 nm (1) and UV 366 nm (2) after development as well as at UV 366 nm (3) and white light (4) after derivatization with vanillin reagent

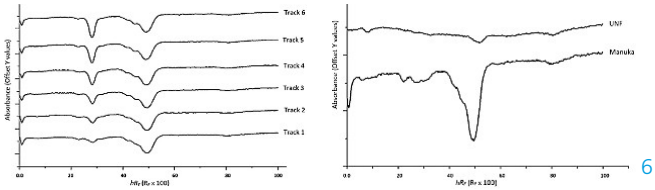
(B) After derivatization, the plate background appeared dark pink, reflecting the color of DPPH* in its reduced state. Where constituents with antioxidant activity reacted with DPPH*, the intensity of the background color was diminished, visualizing compounds with antioxidant zone activity. The stronger the antioxidant activity, the brighter white the active zone appeared against the pink background. Gallic acid was detected on the plate at R_F 0.29 after derivatization.



5

HPTLC chromatograms at white light after DPPH; (left) gallic acid (R_F 0.29) 2.0, 3.0, 4.0, 5.0, 6.0, and 7.0 µL of the standard solution in methanol; (right) Manuka extracts (5 µL) over-spotted with gallic acid 2.0, 3.0, 4.0, 5.0, 6.0, and 7.0 µL respectively

For the quantitative analysis of antioxidant zone activity of honey as gallic acid equivalents, the obtained images were converted into peak profiles (PPI), which were used to derive calibration curves of absorbance *vs* concentration. Using the trend line equations from the calibration curves, the LOD and LOQ were found to be 16.5 ng and 50.0 ng for gallic acid in honey and 14.3 ng and 43.3 ng for pure gallic acid in methanol [3]. As there were no noticeable differences between the LOD and LOQ of gallic acid in methanol and in the honey matrix, it could be concluded that the honey matrix did not have an interfering effect.



6

(left) PPI of a Manuka extract (5.0 µL) over-spotted with 2.0–7.0 µL of gallic acid (R_F = 0.29 ≙ hR_F = 29); (right) PPI of Manuka extract (5.0 µL) and UNF (10.0 µL)

The accuracy of the method (expressed as mean recovery) was within 99.9 to 101.5%. Precision (intra-day and inter-day) and repeatability expressed as standard deviation (SD) and %RSD were between 0.69 and 2.29 (SD) and between 1.01 and 3.07% (%RSD). Repeatability was found to fall within a range of 0.49–1.83 (SD) and 0.60–2.25% (%RSD). The above parameters were all within the International Conference on Harmonization (ICH) guidelines and, thus, confirmed the validity of the method.

The validated HPTLC-DPPH analysis was used to visualize and quantify as gallic acid equivalents the antioxidant activity of individual zones found in Manuka and UNF honey extracts. Four antioxidant zones were detected in Manuka and three active zones in UNF honey extracts.

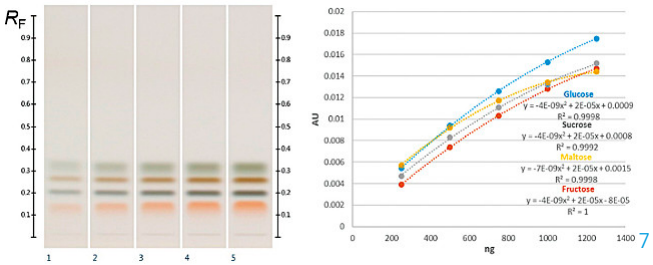
Individual antioxidant zone activity and corresponding R_F in Manuka and UNF honey extracts

	Zones	R_F	Concentration (ng/5 µL extract)	mg equivalent gallic acid per 100 g honey	Total zone activity (mg equivalent gallic acid per 100 g honey)
Manuka	1	0.22	80.32*	0.16	0.97
	2	0.30			
	3	0.48	356.08	0.71	
	4	0.83	47.85	0.10	
UNF	1	0.08	22.02	0.04	0.18
	2	0.51	51.13	0.10	
	3	0.81	18.50	0.04	

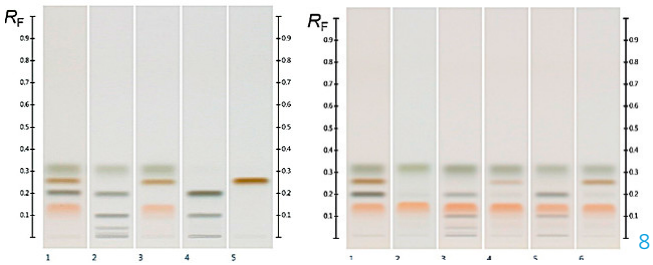
*due to insufficient baseline separation zone 1 and zone 2 were quantitatively accounted for as one capturing an R_F range of 0.19 to 0.36.

(C) At white light individual sugars presented distinct, bright colors: glucose was dark ash, fructose orange, sucrose dark brown and maltose dark green colored. Their corresponding R_F values were for fructose R_F 0.14, maltose R_F 0.20, sucrose R_F 0.27 and glucose R_F 0.32 [5].

All the major sugars in honeys were clearly separated from the matrix and the sugars themselves were clearly separated from each other, forming distinct individual zones. The working range of the evaluated method was determined within the range of 250–1250 ng/zone. The sensitivity of the method (LOD/LOQ) was calculated; LOD/LOQ for glucose: 33.0/100.0 ng; fructose: 22.0 ng/66.6 ng; sucrose: 21.2 ng/64.2 ng; maltose: 63.5 ng/192.5 ng, respectively. The accuracy, precision (intra-day and inter-day), repeatability and robustness of the developed method were also assessed and were within the acceptable ranges outlined in the International Conference on Harmonization (ICH) guidelines.



7 *Fructose (R_F 0.14), maltose (R_F 0.20), sucrose (R_F 0.27) and glucose (R_F 0.32) zones, and their corresponding calibration curves*



8 *(left) HPTLC chromatograms of standards (track 1) and different syrups (from track 2–5: glucose, golden, corn, and maple syrup); (right) standards (track 1), Manuka (track 2) and spiked Manuka (from tracks 3–6 with the respective syrups from the left)*

Honey / Syrup	Sugars within quantification limits				Remarks
	Fructose	Maltose	Sucrose	Glucose	
Manuka	✔			✔	
Jarrah	✔			✔	
Glucose		✔		✔	additional unidentified zones
Golden	✔		✔	✔	
Corn		✔			additional unidentified zones
Maple			✔		

Fructose and glucose contents were readily quantifiable in all honey samples, but maltose and sucrose, if present, were below the LODs in the volumes (3.0 µL) applied (though they can be detected at higher application volumes). In all purposefully adulterated honeys, next to fructose and glucose other sugars like maltose and sucrose were easily quantifiable as they were within the working range.

Thus, mixing honey with sugar syrups leads to significant changes in the amount of sugars such as maltose and sucrose that normally are only present in minor quantities. These changes are

easily detected by HPTLC analysis, as described here, and the method can, therefore, be used not only to quantify major sugars in honey but also as quality control tool to detect sugar syrup adulterations in honey.

Conclusion

(A) HPTLC fingerprint profiling of honey extracts is a quick visual screening method that can also be used for quality control to authenticate a honey's floral origin. The predominant nectar source can be confirmed, additionally unknown or non-specific zones can be investigated for potential additional floral and non-floral compounds, which might assist in detecting adulteration cases. The method is convenient and cost-effective due to the ability to run multiple samples (up to 14) on a single plate.
(B) The study herein used honey as a model matrix to demonstrate the ability of HPTLC-DPPH analysis to visualize and quantify the antioxidant activity of individual constituents in a complex matrix even if their respective chemical identity is not (yet) known. It is anticipated that the approach can also be adopted for the analysis of other matrices with antioxidant activity like plant extracts. The method established here, provides guidance on how to examine other complex systems for potential matrix effects and to capture antioxidant activities of individual constituents.
(C) The HPTLC method for the detection and quantification of simple sugars in honey is easy to perform and offers a convenient approach to not only quantify the major sugars found in honey but also to identify potential adulteration with sugar syrups. The absence of any pre-treatment steps prior to analysis is a major advantage, which might make the method also an interesting analysis approach for the determination of simple sugars in other botanicals and foods.

[1] C. Locher *et al.* J Planar Chromatogr (2017) 30(1):57–62.
[2] C. Locher *et al.* J Planar Chromatogr (2018) 31(3):181–189.
[3] M. K. Islam *et al.* J Planar Chromatogr (2020) 33(3):301–311.
[4] M. K. Islam *et al.* J Planar Chromatogr (2020) 33(5):489–499.
[5] M. K. Islam *et al.* Molecules (2020) 25(22).

Further information is available on request from the authors.

Contact: Dr. Cornelia Locher, CRC for Honey Bee Products and Division of Pharmacy, School of Allied Health, University of Western Australia, Crawley, Western Australia, 6009, Australia, connie.locher@uwa.edu.au

9

CAMAG Derivatizer

Designed to ensure homogeneity and reproducibility, the Derivatizer automatically transfers most of the common derivatization reagents onto thin-layer chromatograms by employing a patented "micro droplet" spraying technology.

To meet the diverging physico-chemical properties of different reagents, *e.g.* acidity and viscosity, four different color-coded spray nozzles are available with six spraying levels to be selected by the user.

In addition to the significantly increased homogeneous reagent distribution, the Derivatizer offers further advantages over manual spraying:

- Environmentally friendly and safe handling through a closed system
- Intuitive handling and easy cleaning
- Low reagent consumption (2–4 mL) through efficient operation
- Reproducible and user-independent results

Further information:
www.camag.com/derivatizer

New Head of Sales & Marketing

My name is Stefan Gaugler and I developed my first analytical methods for CAMAG in 2012 as a method specialist employed by the University of Applied Sciences in Northwestern Switzerland, while I studied analytical chemistry in their master program. When I joined CAMAG in 2014, I took the position as product manager for the DBS-MS 500 (dried blood spot technology). In the following years, we were able to increase sales, build an in-house biosafety II laboratory for DBS method development and advance the knowledge in several application fields of DBS with over ten high impact publications. In addition, I was able to combine my work for CAMAG with a PhD program in analytical chemistry in the group of Dr. Vicente Luis Cebolla Burillo, University of Zaragoza, Spain.

From my first day on, the main hurdle for the acceptance of DBS analysis has been the hematocrit bias. This effect makes absolute quantification of blood ingredients difficult, as we do not know the exact volume of blood analyzed from the dried blood spot. The new version of the DBS-MS 500 to be launched in April 2021 will overcome this substantial limitation and provides the first platform in the market for truly quantitative and fully automated DBS analysis. We are convinced that this new technology will have a big impact on the blood diagnostic market.

Over the years, I was also responsible for project management of several HPTLC projects. The more I worked with HPTLC, the more I started to value its unique features such as on-plate chemistry, the visual comparison of samples on a plate and the fact that you can put anything on the plate without worrying about destroying the stationary phase. Based on my background in HPLC method development, I quickly noticed the many advantages of HPTLC for the characterization of complex mixtures and the power of fingerprinting.

Thrilled by my own experiences, I am highly motivated to share those insights with the world. Together with our experienced sales and marketing team we will provide accessible marketing material such as videos and online tools for a better understanding of the principles and advantages of our technology. We will promote HPTLC and DBS success stories on a global base. Strategies will then be adapted locally through close interactions with distributors and customers, as we must not forget the value of a personal meeting among all those digital tools!

I look forward to the release of new HPTLC PRO modules, as we will do a big step towards digitalization, automation and standardization in the context of industry 4.0. Our future journey will let us explore new possibilities with this sophisticated technology and will lead us into new markets!

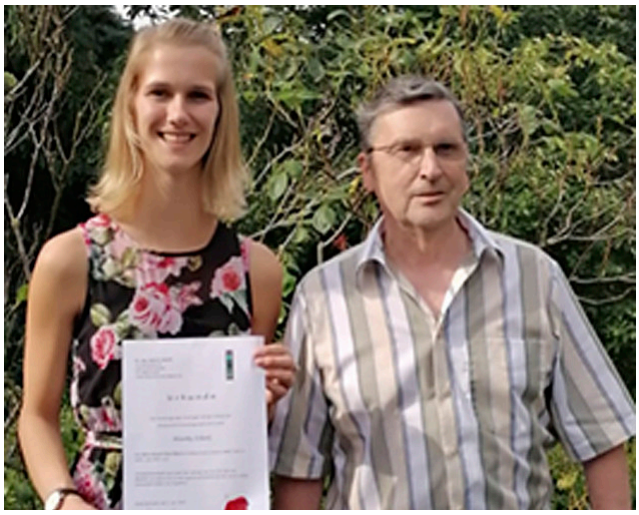
Dr. Stefan Gaugler
Head of Sales & Marketing

Young scientists honored

The next generation

Two bachelor students at the Chair of Food Science, Justus Liebig University Giessen, Germany, with excellent knowledge on HPTLC were decorated with awards for their theses by Elke Hahn-Deinstrop, author of the well-known book Applied Thin-Layer Chromatography: *Best Practice and Avoidance of Mistakes*, and by Dr. Heinz Hauck, who led the HPTLC research at Merck for more than 3 decades.

Awardee Sophie Arnold honored by Elke Hahn-Deinstrop



Awardee Mareike Schenk honored by Dr. Heinz Hauck

Sophie Arnold worked on the development of a quantitative trace analysis method for the analysis of androgenic and anti-androgenic substances migrating from food packaging into food.
Together with the doctoral student Daniel Meyer, she exploited the newly developed planar yeast androgen/anti-androgen screen (pYAAS) [1]. It is a new concept that can be applied to any assay and detects agonist/antagonist activities at the same time. In order to apply the new HPTLC-pYAAS assay concept in trace analysis, she investigated different mobile phases, device settings and confounding factors like the impact of humidity and cell agglomeration. With regard to quantification, different programs and settings were investigated for the evaluation of negative peaks obtained by fluorescence measurement. She investigated also first parameters of method validation.

Mareike Schenk developed specific HPTLC metabolite profiles for characterization and identification of probiotic bacterial strains in complex samples like feed. Together with the doctoral student Stefanie Kruse, she had to identify differences in the metabolite profiles of the selected strains based on previous work [2]. The influence of cultivation parameters on the bacterial metabolite profile was investigated, including different culture media, cultivation times and oxygen levels. The impact of heat and ultrasound treatment on the HPTLC metabolite profiles was also studied.
Different extraction solvents and HPTLC parameters were investigated to obtain specific metabolite profiles. Derivatization reagents were applied for the functional characterization of individual metabolites and their quantification via the standard addition method.

[1] Klingelhöfer, I., Hockamp, N., Morlock, G.E., Anal. Chim. Acta, 1125 (2020) 288
[2] Kruse, S., Pierre, F., Morlock, G.E., J Chromatogr. A, 1640 (2021) 461929

Universal HPTLC Mix: the rise of a novel concept for system suitability test

From left: Dr. Tiên Do, Dr. Marco Schmid, Dr. Eike Reich, Dr. Manjusha Phanse, Akshay Charegaonkar

The idea of developing a universal system suitability test (SST) for HPTLC originated at the company Anchrom (India), where Dr. Manjusha Phanse had started the evaluation of the concept. Thinking about the practicality of qualifying an HPTLC analysis and the needs of clients for routine analysis, the laboratory teams of CAMAG and Anchrom worked together to create a new SST concept for HPTLC. This project was later supported by Sigma-Aldrich Chemie GmbH (subsidiary of Merck KGaA, Germany). The outcome was a joint publication in the Journal of Chromatography A [1].

Introduction

An SST is commonly used in routine quality control to validate the performance of an analytical system, including the method and the apparatus. In chromatography, the SST is a process that analyzes the behavior of specific reference substances under certain chromatographic conditions to know whether the method is reproducible, robust, and suitable for the intended application.
In HPTLC, the SST often qualifies only a limited region of the chromatogram (*e.g.*, specific R_F values or small R_F ranges due to the need for having barely separable substances). If no deviation from the acceptance criteria is observed, the entire chromatographic system is considered compliant. However, in practice, the chromatographic quality of the other regions remains unknown. Additionally, HPTLC methods using developing solvents of different polarity and selectivity may require different sets of substances for SST. Some substances are costly and not readily available, which can increase the cost of analysis. To overcome these problems, a Universal HPTLC Mix (UHM) for use in SST was developed [1].

With the UHM, HPTLC laboratories have a single solution, applicable as SST to a wide range of chromatographic systems, with different polarities and selectivities. Its low price, stability in solution, and capability to detect small chromatographic variations make the UHM particularly attractive. The replacement of conventional substances for SST by the UHM will help laboratories to save time and money required for laborious investigation of specific reference substances for each method to be qualified. Different fields of application can benefit from the UHM concept, such as herbal drugs, forensics, pharmaceuticals, cosmetics, etc.

Standard solutions

Sulisobenzone, thymidine, paracetamol, 9-hydroxyfluorene and 2-(2H-benzotriazol-2-yl)-4-(1,1,3,3-tetramethylbutyl)phenol were prepared at 1 mg/mL. Guanosine is prepared at 0.5 mg/mL, thioxanthen-9-one at 0.01 mg/mL, and phthalimide at 2 mg/mL. All substances are dissolved in methanol.

Chromatogram layer

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

Sample application

2.0 µL of 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 are developed 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. 20 different developing solvents (eight of them are listed below) were investigated, followed by drying for 5 min.

N°	Development solvent	Polarity index	Selectivity groups
A	Ethyl acetate, formic acid, acetic acid, water 100:11:11:26 (*V/V*)	5.63	VI, IV, VIII
B	Ethyl acetate, formic acid, water 15:1:1 (*V/V/V*)	4.76	VI, VIII
C	Dichloromethane, methanol, water 14:6:1 (*V/V/V*)	4.01	V, I, VIII
D	Toluene, acetic acid 4:1 (*V/V*)	3.12	VII, IV
E	Toluene, ethyl acetate 3:1 (*V/V*)	2.90	VII, VI
F	Toluene, ethyl acetate 9:1 (*V/V*)	2.60	VII, VI
G	Toluene, methanol, diethylamine 8:1:1 (*V/V/V*)	2.58	VII, I
H	Cyclohexane, ethyl acetate 5:3 (*V/V*)	1.73	VI

Note: Formic acid is neither included for the polarity index calculation nor the selectivity group as there are none defined in the literature, to the best of our knowledge. Diethylamine is not included in a selectivity group.

Documentation

Images of the plate are captured with the TLC Visualizer 2 at UV 254 nm and 366 nm.

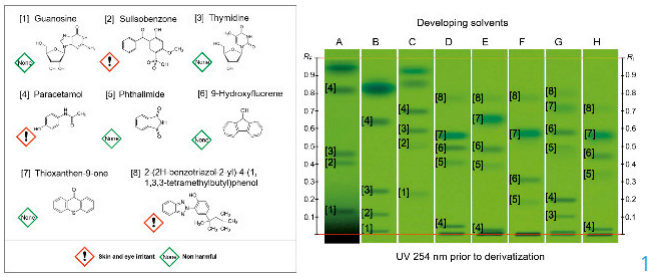
Densitometry

Absorbance measurement at 254 nm and fluorescence measurement at 366 nm with TLC Scanner 4 and *visionCATS*, slit dimension 5.00 mm x 0.20 mm, scanning speed 20 mm/s. For the fluorescence measurement, a mercury lamp and a cut-off filter at 400 nm are used.

Results and discussion

In the first step of the investigation, the suitable substances for the UHM were selected. The researchers [1] considered the following criteria, which lead to a list of 56 candidates: 1) Low hazard (not harmful and non-toxic substances); 2) Detectability at UV 254 and 366 nm prior to derivatization; 3) Stability in solution for at least two months; 4) Low cost (<50 CHF/g).

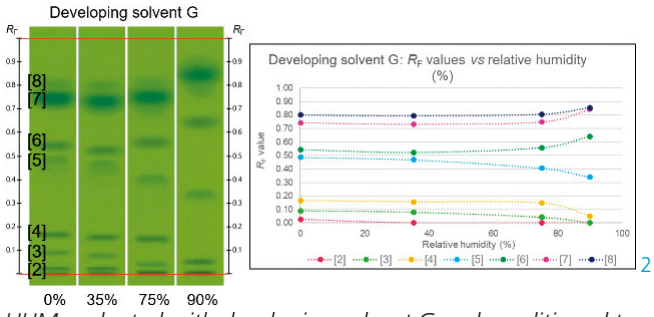
The chromatographic behavior of those 56 standards was evaluated with 20 developing solvents, covering a wide range of polarity and selectivity. The objective was to find a smaller group of substances that achieves an even distribution throughout the entire chromatogram for the maximum number of different developing solvents. Additionally, each developing solvent should achieve baseline separation for at least 3–4 substances. The chosen substances and their fingerprints in eight different developing solvents are shown in the following image.



1

Substances selected for UHM and the HPTLC fingerprints of the UHM in eight different developing solvents

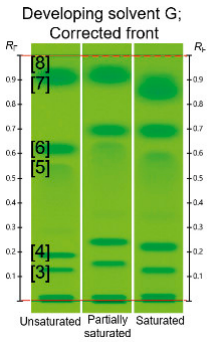
To evaluate, whether the UHM responds to variations of the chromatographic conditions, three experiments were performed. In the first, plates were conditioned to different relative humidity (from 0% to 90%) prior to development. As shown in the image below, the UHM is sensitive to variations in relative humidity, particularly to the higher ones. The differences are more expressive if the developing solvent contains no water.



2

UHM evaluated with developing solvent G and conditioned to different relative humidities prior to development

In the second experiment, the individual proportion of the solvents in developing solvents B and F was changed (±10%), and the effect on the chromatography was evaluated. A difference of up to 0.06 R_F units was observed from the mean R_F values of the control track. In the third experiment, different levels of chamber saturation were tested: unsaturated, partially saturated (20 min, no filter paper), and saturated (20 min, with filter paper). R_F values increased with partial saturation, but then decreased with full saturation, proving that the UHM can detect chamber saturation problems.



3

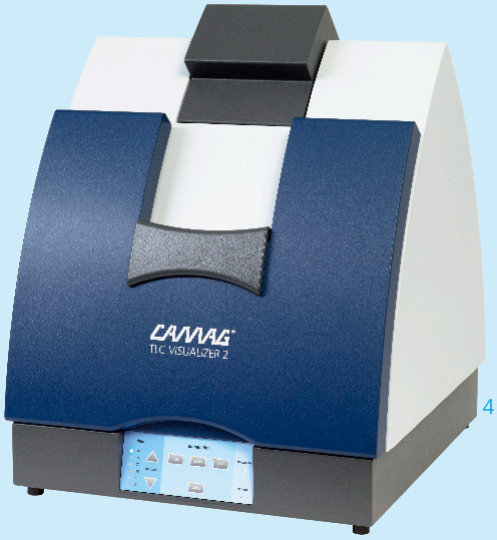
UHM evaluated with developing solvent G developed with different levels of chamber saturation

The UHM performance was evaluated in intra- and inter-laboratory tests based on the ΔR_F in developing solvents B, F and G. For the intra-laboratory test, the confidence interval ΔR_F was 0.03, while for the inter-laboratory test, this value was 0.04.

[1] 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). DOI: 10.1016/j.chroma.2020.461830.

Further information is available on request from the authors.

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



4

CAMAG TLC Visualizer 2

High-end imaging and documentation system for TLC/HPTLC chromatograms

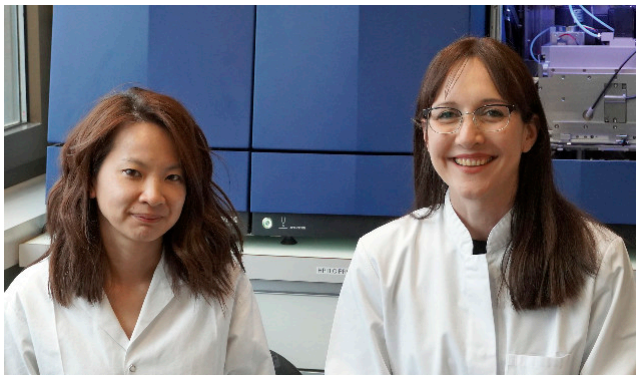
The visual presentation of the complete chromatogram with all samples and standards side by side is one of the most convincing arguments for TLC/HPTLC – and this is precisely what the TLC Visualizer 2 is designed for.

Powered by *visionCATS* HPTLC software, the high-end imaging and documentation system reproducibly acquires and preserves best quality images of TLC/HPTLC chromatograms under different illuminations. The obtained images can be separated into tracks representing single samples, whereas the »Comparison Viewer« tool allows for creating virtual plates from tracks originating from different plates, *e.g.* for batch-to-batch comparison or long-term stability testing. The integrated USB 3.0 port ensures easy PC connection and high-speed data transmission.

The TLC Visualizer 2 meets all requirements for operation in a cGMP/cGLP environment.

Further information:
www.camag.com/tlc-visualizer2

Fast analysis of sugars in honey by using the HPTLC PRO System



From left: Dr. Tiên Do, Dr. Melanie Broszat

CAMAG introduced the concept for the new modular HPTLC PRO System in 2019. Since then, CAMAG's HPTLC Laboratory transfers existing methods and develops new methods for this System. Dr. Melanie Broszat and Dr. Tiên Do are intensively working on this topic while staying in close exchange with the entire lab team and the research & development department.

Introduction

The quantification of simple sugars can be challenging due to their high polarity, low volatility, their lack of a chromophore and their common occurrence in complex matrices [1–3]. HPTLC can separate mono- and oligosaccharides after minimal sample preparation and can sensitively detect these compounds after post-chromatographic derivatization. The published method for quantification of sugars in honey [2] allows analyzing multiple samples on a single plate within approximately 3.7 hours. With the method transferred to the new HPTLC PRO System, this test can be accomplished in about 2.5 hours. An alternative method developed for HPTLC PRO requires just 1.3 hours per plate.

HPTLC allows quantification of sugars in honey and other complex matrices at low running costs. Depending on the level of equipment used, the speed, automation and reliability of the obtained quantitative results can be increased. With the new method developed for the HPTLC PRO System, the main sugars in honey can be investigated in short time and other sugars, such as oligomers present in fermentation processes, can be analyzed at the same time.

Standard solutions

Individual sugars are dissolved in 50% aqueous acetonitrile with sonication to obtain a final concentration of 1.0 mg/mL for qualitative tests, method transfer and method development. For quantification and during determination of the working range, a mixture of fructose, maltose, sucrose, and glucose at concentration levels between 12.5 µg/mL–1000.0 µg/mL is used in 50% aqueous acetonitrile.

Sample preparation

The samples are dissolved in 50% aqueous acetonitrile with sonication to obtain a final concentration of 1.0 mg/mL for qualitative tests and are applied in 20-fold dilution for quantification of the two main sugars in honey (fructose, glucose).

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 Automatic TLC Sampler (ATS 4, quantitative settings, 10 µL syringe) or the HPTLC PRO Module APPLICATION using the default settings (two rinsing solutions), 20 tracks, band length 6.0 mm, distance from left edge 18.0 mm, track distance 8.5 mm, distance from lower edge 8.0 mm. 1.0–3.0 µL for sample solutions and 1.0 µL for standard solutions are applied.

Chromatography

(**1**) Plates are developed in the ADC 2 with chamber saturation (with filter paper, 60 min), after activation at 33% relative humidity (*) for 10 min using a saturated solution of magnesium chloride, followed by 5 min pre-conditioning, development with *n*-butanol – isopropanol – aqueous boric acid

(5 mg/mL) 3:5:1 (*V/V*) to the developing distance of 85 mm (from the lower edge), followed by drying for 15 min** [2].
(**2**) Plates are developed in the HPTLC PRO Module DEVELOPMENT after activation at 0% relative humidity (*molecular sieve) for 10 min, followed by 90 s pre-conditioning at 30% pump power, development with *n*-butanol – isopropanol – aqueous boric acid (5 mg/mL) 3:5:1 (*V/V*) to the developing distance of 70 mm (from the lower edge), followed by drying for 15 min.
(**3**) Plates are developed in the HPTLC PRO Module DEVELOPMENT after activation at 0% relative humidity (molecular sieve) for 10 min, development with ethyl acetate – methanol – boric acid (5 mg/mL) – acetic acid 50:40:10:2 (*V/V*) to the developing distance of 70 mm (from the lower edge), followed by drying for 5 min.
*Note: *methods (1) and (2) are very robust and no significant differences for the R_F values were obtained between 0 and 33% relative humidity; **deviation from [2]*

Post-chromatographic derivatization

Aniline-diphenylamine-phosphoric acid reagent (ADPA reagent): 2.0 g of diphenylamine and 2.0 mL of aniline are dissolved in 80.0 mL of methanol, 10.0 mL of *o*-phosphoric acid (85%) are added and the mixture is shaked until any precipitate is dissolved, then again 10.0 mL of methanol are added. The plate is sprayed with the Derivatizer (yellow nozzle, spraying level 6), heated at 110 °C for 10 min on the TLC Plate Heater.

Documentation

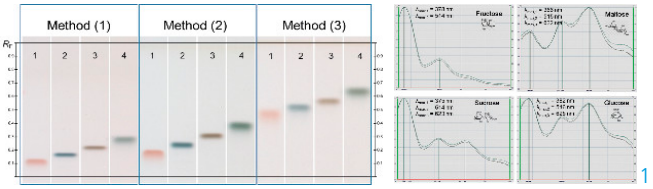
Images of the plate are captured with the TLC Visualizer 2 at UV 366 nm and white light after derivatization.

Densitometry

Absorbance measurement at 370 nm [3] is performed with TLC Scanner 4 and *visionCATS* 3.0 (slit dimension 5.00 mm x 0.30 mm, scanning speed 50 mm/s, data resolution 25 µm/step for single-wavelength scan, spectra recording from 350–800 nm).

Results and discussion

The methods have been compared for their consumption of time and consumables, and their repeatability: method (**1**) with the conditions from [2] by using the ATS 4 and ADC 2, method (**2**) with the developing solvent from [2] by using the HPTLC PRO Modules APPLICATION and DEVELOPMENT, and method (**3**) with an alternative developing solvent by using the HPTLC PRO Modules APPLICATION and DEVELOPMENT. All three methods are well suited for quality control of honeys.



HPTLC chromatograms at white light after derivatization with ADPA reagent: tracks 1–4 standards fructose, maltose, sucrose, and glucose with increasing R_F; UV/Vis spectra from 350–800 nm (recorded on the plate obtained with method (3))

The UV/VIS spectra recorded after derivatization show a high signal response for all analytes at 370 nm. Therefore, LODs/LOQs have been determined for the four relevant sugars in honey at this wavelength to facilitate evaluation in routine quality control by using scanning densitometry at a single wavelength ($LOD_{370\,nm}$/$LOQ_{370\,nm}$ for fructose and sucrose: 6.0/18.0 ng/zone, for maltose and glucose: 12.0/48.0 ng/zone). The linear working range extends from $LOQ_{370\,nm}$ to 125.0 ng/zone.

Comparison of time and consumables required for each of the three methods

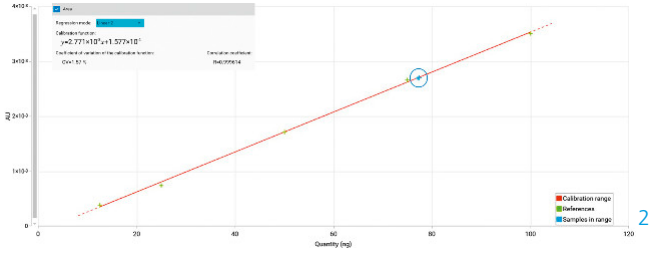
	HPTLC (**1**)	HPTLC PRO (**2**)	HPTLC PRO (**3**)
Time for application of 20 tracks	~ 39.0 min (ATS 4 quantitative settings)	~ 39.0 min	~ 39.0 min
Developing time per plate	~ 101.0 min	~ 84.0 min	~ 21.0 min
Total run time per plate (incl. time for application, saturation, pre-conditioning, drying)	~ 220.0 → 11.0 min/track	~150.0 → 7.5 min/track	~ 76.5 → 3.8 min/track
Solvent consumption per plate	~ 70.0 mL → 3.5 mL/track	~ 80.0 mL → 4.0 mL/track	~ 65.0 mL → 3.3 mL/track

For the quantification of fructose, maltose, sucrose, and glucose, method (**2**) is recommended. In this case, the best separation of the four analytes in an optimum R_F range is achieved in significantly less run time compared to method (**1**). To proof the suitability of the method (**2**) for quantification, four samples of honey have been selected of which one was mixed with maple syrup 1:1 to determine the recovery. The results are listed in the following table.

Sugar concentration in the selected samples obtained with method 2

	Concentration (g/100 g)			
	F	*G*	*M*	*S*
Maple syrup	*n.d.*	*n.d.*	*n.d.*	67.67
Maple syrup + wild bee honey	31.97	22.42	6.13	68.59
Wild bee honey	31.88	24.86	6.96	*n.d.*
Linden blossom	43.49	22.02	4.49	*n.d.*
Honeydew	40.82	35.48	6.46	*n.d.*

F: fructose, G: glucose, M: maltose, S: sucrose; n.d.: Not detected



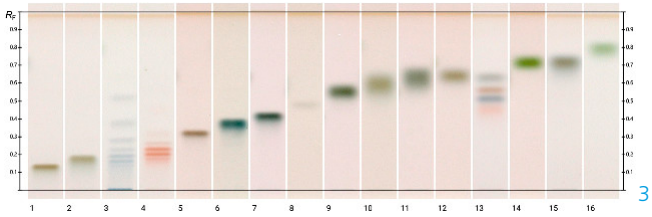
2

Calibration curve of sucrose (method 2); blue circle shows the amount detected in the samples maple syrup and wild bee honey mixed with maple syrup.

Comparison of R_F values of selected standards and ΔR_F of relevant sugars in honey from different plates and/or from different days (n=3 for method (1), n=6 for methods (2) and (3))

	R_F HPTLC (**1**)	R_F HPTLC PRO (**2**)	R_F HPTLC PRO (**3**)
Raffinose	*0.05*	*0.08*	*0.32*
Maltotriose	*0.07*	*0.11*	*0.37*
Lactose	*0.10*	*0.15*	*0.42*
Fructose	***0.12 ± 0.03***	***0.18 ± 0.04***	***0.47 ± 0.02***
Trehalose	*0.15*	*0.21*	*0.48*
Maltose	***0.16 ± 0.03***	***0.24 ± 0.03***	***0.52 ± 0.01***
Galactose	*0.20*	*0.29*	*0.55*
Ribose	*0.20*	*0.29*	*0.60*
Sucrose	***0.22 ± 0.04***	***0.31 ± 0.03***	***0.56 ± 0.03***
Arabinose	*0.27*	*0.37*	*0.64*
Glucose	***0.28 ± 0.04***	***0.38 ± 0.04***	***0.63 ± 0.02***
Mannose	*0.30*	*0.40*	*0.63*
Fucose	*0.34*	*0.45*	*0.71*
Rhamnose	*0.47*	*0.59*	*0.81*

Method (**3**) is best suited for the analysis of sugars of different sizes (mono- and oligomers) and sugar acids of high polarity (*e.g.* glucuronic acid). The entire migration distance is used for separation whereas methods (**1**) and (**2**) are optimized for the separation and quantification of mono- and dimers.



3

HPTLC chromatograms of different standards (method 3) at white light after derivatization; track 1: galacturonic acid, 2: glucuronic acid, 3: maltodextrin, 4: fructo-oligosaccharides, 5: raffinose, track 6: maltotriose, 7: lactose, 8: trehalose, 9: galactose, 10: ribose, 11: mannose, 12: arabinose, 13: mixture of fructose, maltose, sucrose, and glucose (250 ng each), 14: fucose, 15: xylose, 16: rhamnose (1 µg each, except for the mixture on track 13)

Conclusion

With the three methods described herein, the principal sugars of honey can be analyzed at low running costs. The instrument investment costs for method (**1**) are lower, but more time and manual intervention is required for each analysis. Methods (**2**) and (**3**) have been developed for routine quality control and a high sample throughput. In this case, the level of automation and reduced time per sample are of greater importance, making the HPTLC PRO System the better choice. Method (**3**) can be used for sugar analysis in general, *e.g.* for optimization and monitoring of fermentation processes and for analysis of sugar containing products in divers matrices.

[1] M. K. Islam *et al.* J Planar Chromatogr (2020) 33(5):489–499

[2] M. K. Islam *et al.* Molecules (2020) 25(22)

[3] G.E. Morlock, G. Sabir, J Liquid Chromatogr (2011) 34:902–919

Further information is available on request from the authors.

Contact: Dr. Melanie Broszat, CAMAG, Sonnenmattstrasse 11, 4132 Muttenz, Switzerland, melanie.broszat@camag.com

CAMAG® HPTLC PRO Module PLATE STORAGE – The key to autonomous processing of up to five HPTLC plates

1

The Module PLATE STORAGE is the key to sequential application and development of several plates. It feeds the HPTLC PRO System with clean plates and stores the processed plates during or after analysis. Unlike the present HPTLC workflow, employing this module avoids any manual intervention and thus reduces the human factor to the minimum, resulting in the highest analytical quality, in maximum reproducibility and in great handling convenience.

The operator's tasks are now limited to preparing the samples and required solvents, filling the stacker with clean plates before the start, and removing the processed plates at the end of the process.

To avoid cross contamination of plates, the Module PLATE STORAGE features a fume extraction system for the active suction of vapors from the stacker holding the processed plates.

Integrated in *visionCATS* 3.1, the latest release of the CAMAG HPTLC software covering all HPTLC instruments as well as the HPTLC PRO System, the Module PLATE STORAGE allows to run a sequence of HPTLC analyses autonomously overnight.

Module PLATE STORAGE:
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