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ISSUE 1 | 2022

Ensuring product safety and quality with HPTLC

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- Quantification of methylglyoxal in Manuka honey
- Screening for chlorinated paraffins in vegetable oils
- Quantification of glucose degradation products
- The HPTLC Association Substance Database
- HPTLC detection of falsification in drugs

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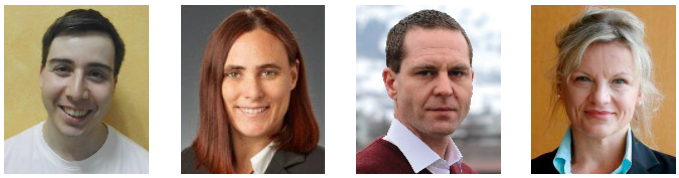
Quantification of methylglyoxal in Manuka honey – A simple HPTLC based approach



From left: Markus Burholt, Michaela Oberle, Michael Schulz, and Dr. Monika Bäumle (Merck)

Introduction

Honey is one of the most frequently tested natural food products. In recent years, Manuka honey has gained popularity because of its high antibacterial activity [1]. Methylglyoxal (MGO) was identified as one of the major contributors to its antibacterial property. MGO is present in high concentrations in manuka honey and is directly responsible for its potency. This makes the Manuka honey exclusive and high-priced as compared to the other traditional kinds of honey. Manuka honey from New Zealand usually contains 40 to 800 mg/kg of MGO but can even contain up to 1900 mg/kg [2]. To avoid adulteration of Manuka honey products, a strict quality regulation regarding its origin, purity, and quality needs to be fulfilled and is a prerequisite for the UMF™ (Unique Manuka Factor) grading [2]. It mostly reflects the MGO amount in the honey but also considers other authenticity markers.

In the following application, we focus on the MGO quantification using HPTLC with subsequent substance confirmation by MS measurement. The high viscosity and high sugar content of honey make it a very complex and matrix-rich sample to analyze. HPTLC is a convenient, fast, and efficient separation technique that enables the development of analytical methods without the need for complicated sample preparations or high investments [3]. Low cost and short analysis time per sample are given by the parallel analysis of many samples on one plate. Furthermore, the high matrix tolerance of HPTLC offers additional opportunities to existing routine methods.

Six different commercially available Manuka honey samples were analyzed. MGO shows a mesomeric effect and reacts immediately with water to form either methylglyoxal monohydrate or methylglyoxal dihydrate [4]. Only a small amount of around 1% MGO remains unreacted. Direct detection of MGO in Manuka honey is difficult with conventional methods. In this application, MGO is converted to the stable 2-methylquinoxaline by derivatization with 1,2-phenylenediamine [5]. The stable form is then used as the reference. For confirmation of the method and determination of the recovery rate,

regular honey samples were spiked with MGO and 1,2-phenylenediamine. Other derivatization options were tested but the reaction with 1,2-phenylenediamine performed best. Water and honey matrix were tested, to confirm that the optimized reaction conditions provide reproducible results for both matrices.

Standard solutions

The standards are prepared by dissolving 100 µL of Sigma-Aldrich ~40% aqueous MGO solution (exact content known from batch specification) in 20.0 mL of water. 800 µL of the stock solution is further diluted with water to 10.0 mL and 0.2% (2 mg/mL) of the reactant Supelco 1,2-phenylenediamine powder is added. All standard solutions are stored at 8°C for two days before use to achieve reproducible reaction of MGO with 1,2-phenylendiamine. Longer storage time (>3 days) leads to partial degradation of 2-methylquinoxaline.

Sample preparation

Honey samples are prepared at 100–150 mg/mL (in the shown examples: honey sample solutions of 100 mg/mL in case of sample numbers 1, 3, 5, and 150 mg/mL in case of honey samples 2, 4 and 6 were applied in a higher volume due to the expected lower amount of MGO). To each sample 0.2% of 1,2-phenylenediamine is added, *e.g.*, sample 1, 4.0 g honey diluted in 40 mL solution of water/ethanol in 3:2. To the solution, 0.2% (2 mg/mL) of the reactant 1,2-phenylenediamine is added. Before using the samples, they need to be stored at 8°C for two days to complete the reaction.

Chromatogram layer

Supelco HPTLC silica gel 60 F_{254} 20 x 10 cm are used. The plates are pre-washed with the developing solvent (up to 70 mm) and dried before use.

Sample application

Between 0.3–9.0 µL of the samples and standard solutions are applied as areas 5 x 3 mm [2] with the ATS 4 (y = 10 mm, x = 15 mm). This step is necessary because of the high matrix and high application volumes of the honey samples.

Chromatography

After drying, the plate is developed in a Twin Trough Chamber (20 x 10 cm, both troughs filled, 15 minutes waiting time before use, without filter paper) with ethyl acetate – acetonitrile 85:15 (*V/V*) to the migration distance of 50 mm (from the lower edge), followed by drying for 5 min at 60°C with a plate heater.

Post-chromatographic derivatization

After development, the plate is dried and then derivatized by dipping in an anisaldehyde-sulfuric acid reagent (0.5 mL *p*-anisaldehyde, 85 mL methanol, 10 mL glacial acetic acid, 5 mL sulfuric acid 98%). Blue zones of 2-methylquinoxaline (product of the reaction of MGO with 1,2-phenylenediamine) appear at R_F 0.8. The yellow zone at R_F 0.7 is unreacted 1,2-phenylendiamine.

Documentation

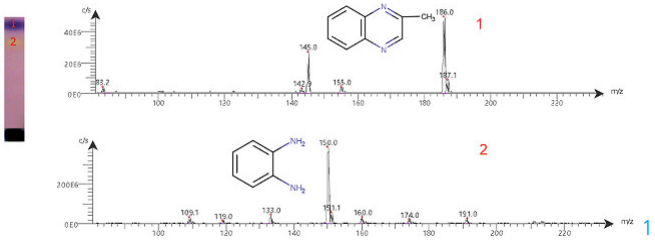
Images of the plate are captured with the TLC Visualizer in white light.

Densitometry

Absorbance measurement at 480 nm is performed with CAMAG TLC Scanner 3 (slit dimension: 2.00 x 0.20 mm, scanning speed: 20 mm/s, data resolution: 100 µm/step).

Mass spectrometry

The target zones are marked in UV light 254 nm. Afterwards, they are directly eluted from the plate with acetonitrile – water – formic acid 95:5:0.1 (*V/V*) and an MS-Interface, and measured with a single quadrupole MS.



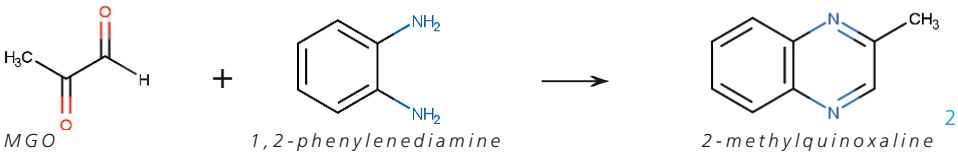
Mass spectra of 2-methylquinoxaline (m/z = 145.0 [M+H⁺] and 186.0 [M+ACN+H⁺]) and 1,2-phenylendiamin (m/z = 150.0 [M+ACN+H⁺])

Results and discussion

As demonstrated, MGO can be identified and quantified in different honey samples within the concentration range of 50 to 600 mg/kg. The conversion of MGO into the more stable com-

pound 2-methylquinoxaline allows for an easy evaluation of the MGO content.

A recovery study was performed using regular honey to simulate the honey matrix. It was spiked with a known amount of MGO standard solution, followed by the addition of 1,2-phenylenediamine. The measured (and calculated) MGO amount allowed for the correlation of the actual amount of MGO in the Manuka honey samples. The recovery study showed a detectable MGO amount of around 90%. The correlated MGO amount in Manuka samples was calculated accordingly. One of the samples (sample 1) showed a lower MGO content than indicated by the supplier. This might be because of the degradation of the MGO during storage. Samples 2 and 6 only showed MGO concentrations of 50 and 100 mg/kg. These Manuka honey samples are considered of lower quality; although no MGO concentration was provided by the supplier.

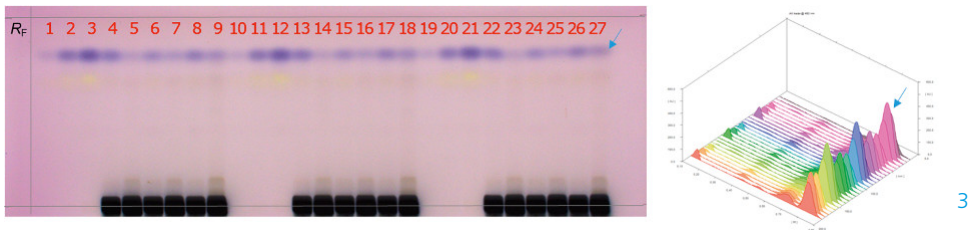


MGO 1,2-phenylenediamine 2-methylquinoxaline 2

Reaction mechanism of MGO and 1,2-phenylenediamine

Table with obtained results

Manuka Samples	Application Position	Conc. Samples mg/ml	Application volume µl	Mean Area AU	Mean Amount µg	RSD %	MGO in Honey mg/kg	Expected Amount MGO in Honey mg/kg
1	4, 13, 22	100.0	5.0	11225	0.228	2.68	507.4	600.0
2	5, 14, 23	150.0	9.0	4548	0.055	2.84	45.3	~50.0*
3	6, 15, 24	100.0	5.0	8002	0.136	3.10	301.4	300.0
4	7, 16, 25	150.0	8.0	6031	0.088	2.48	81.4	80.0
5	8, 17, 26	100.0	5.0	9654	0.181	3.06	401.8	400.0
6	9, 18, 27	150.0	8.0	7578	0.125	3.14	115.6	~100.0*



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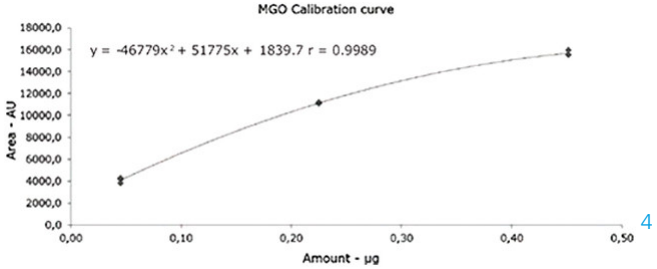
HPTLC chromatograms in white light after derivatization with anisaldehyde (left) and the corresponding densitogram (right) measured at 480 nm (zones at R_F 0.80)



4

Calibration curve for quantification of MGO in Manuka honey samples

In summary, the analysis of MGO in a complex and challenging food matrix like honey was described. The target analyte could be easily separated and detected without time-consuming and labor-intensive sample preparation. The flexible set-up enabled a combination with MS measurements. Screening and method development capabilities were shown by the application of 27 tracks on one plate. The study clearly differentiated various honey qualities (referring to MGO content) on the market. Though the analysis of MGO is challenging, MGO content could be well quantified in the expected range. A fast, cheap, and simple quantification of MGO can be accomplished with HPTLC.

[1] E. Mavric, *et al.* (2008) Mol Nutr Food Res

[2] https://newzealandhoneyco.com/pages/mgo-vs-umf-calculator-manuka-honey

[3] M. Schulz *et al.* (2009) Analytix Reporter, Issue 5

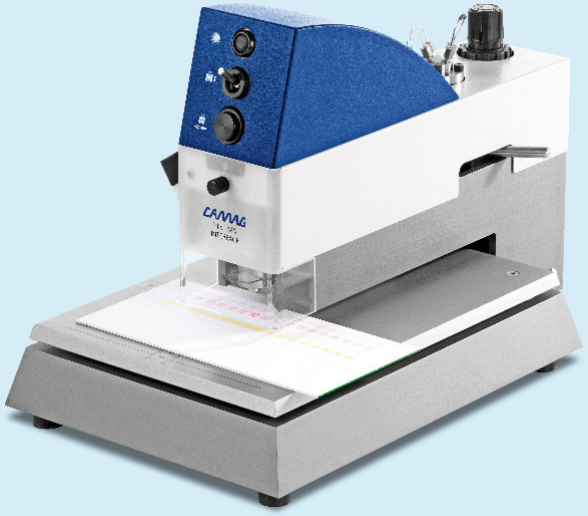
[4] https://magritek.com/2021/01/29/identification-and-quantification-of-methylglyoxal-in-manuka-honey/

[5] E. Dimitrova (2013), Master thesis, TU University of Graz, Austria

[6] Merck (unpublished results)

Further information is available on request from the authors.

Contact: Dr. Monika Bäumle, Global Product Manager TLC, Merck, monika.baeumle@merckgroup.com.



5

CAMAG® TLC-MS Interface 2

The elution-based TLC-MS Interface 2 is a highly convenient and versatile instrument for the rapid elution of TLC/HPTLC zones with direct transfer to a mass spectrometer. It can be installed plug & play with any LC-MS system without adjustments or mass spectrometer modifications. Depending on the MS system, a substance can be identified within a minute via its mass spectrum, or for an unknown substance zone, the respective sum formula can be obtained. Furthermore, interesting zones can be eluted into vials for further investigations with, *e.g.*, NMR, (ATR-) FTIR, ESI-MS, and MALDI-MS.

Key features:

- Rapid and contamination-free elution of selected zones
- Direct transfer to any mass spectrometer
- Plug & play installation
- Confirmation of known substances within a minute
- Any plate format up to 20 x 20 cm

Further information:
www.camag.com/tlc-ms2

Anchrom – CAMAG's distributor in India

Anchrom Enterprises (I) P. Ltd, our distributor in India for more than 40 years, very actively promotes HPTLC. One such activity, started in 2006, is to award the best research publications from India, which used HPTLC. Three awards for 1st, 2nd and 3rd are given in the two research categories private industry (category I) and government and education institutes (category II). Often an award is jointly shared.

Dr. P. D. Sethi (1935-2015)

Late Dr. P.D. Sethi was one of India's topmost pharmaceutical analysts and Director of the Central Indian Pharmacopeia Laboratory. In that capacity, in the late 1980s, he quantitatively analyzed hundreds of family planning tablet batches by HPTLC. He has written several books on HPTLC, which are widely used in India.

The prize money is INR 35000, 25000, and 15000 for the 1st, 2nd and 3rd awardee in each of the two categories. For the calendar year 2020, 12 papers were selected by the external "Awards Evaluation Committee", seven from institutes and five from industry.

On Dec 17, 2021, one author from each of the 12 award winners was invited to the Head Office in Mumbai for the award presentation. Thus, 12 top HPTLC users of India met and exchanged views. In Anchrom's HPTLC Applications Research Center, they were taken around the lab and shown various specialized instruments like the next generation HPTLC PRO Modules, HPTLC-MS, Derivatizer, AMD 2 for gradient HPTLC, and *visionCATS* software etc.

Dr. Rahul Singh, First prize, category I (award presented by Rutvika Charegaonkar, CFO, Anchrom)

Dr. Rahul Singh was awarded the 1st prize for his excellent work on the role of HPTLC in the manifestation of individual ingredients of herbal formulations and the development of a rapid, sensitive, and reproducible HPTLC method for one of the major bioactive markers, piperine. At Emami Ltd, a top Indian FMCG company, Dr. Rahul Singh developed in the last decade several hundred methods for HPTLC fingerprinting and applies them daily in QC.

Dr. Shrikrishna Nandanwadkar, First prize, category II (award presented by Akshay Charegaonkar, Director, Anchrom)

Dr. Nandanwadkar, a young scientist, won the 1st prize in category II for his path breaking work in detection of carcinogenic aryl azo dyes in foods by HPTLC and MS. Previously, such carcinogens were analyzed only in textile and leather dyes.

Award winners with Anchrom team

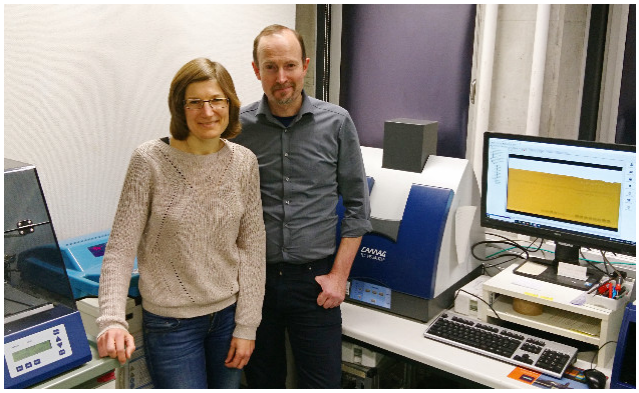
The event was a real success and demonstrated once more how convenient and versatile HPTLC can be used. CAMAG and Anchrom congratulate all award winners and we are looking forward to the next *Sethi Memorial National Award 2021*.

List of Dr. Sethi Award Winners 2020

	Government/Education Sector Winners
A	**1st Prize**
1.	Authors: Dr. Shrikrishna Nandanwadkar *et al.* Name of Institute: KLE's College of Pharmacy, Belagavi Title of Paper: A novel digitally optimized rapid quantification of carcinogenic aryl azo amines from various food matrices by HPTLC-MS
B	**Joint 2nd Prize**
1.	Authors: Dr. Pallavi Choudhary *et al.* Name of Institute: Regional Forensic Science Laboratory, New Delhi Title of Paper: HPTLC–MS method for the determination of benzodiazepines in urine samples
2.	Authors: Dr. Karuna Shanker *et al.* Name of Institute: CSIR – Central Institute of Medicinal and Aromatic Plants, Lucknow Title of Paper: HPTLC method for the simultaneous determination of six bioactive terpenoids in *Putranjiva roxburghii* Wall
3.	Authors: Dr. Pintu Prajapati *et al.* Name of Institute: Maliba Pharmacy College, Tarsadi Title of Paper: Implementation of QRM and DoE-Based Quality by Design Approach to VEER Chromatography Method for Simultaneous Estimation of Multiple Combined Dosage Forms of Paracetamol
C	**Joint 3rd Prize**
1.	Authors: Dr. Sharad Srivastava *et al.* Name of Institute: Pharmacognosy Division, CSIR National Botanical Research Institute, Lucknow Title of Paper: Simultaneous HPTLC-UV quantification of colchicine and gloriosine alkaloids in the natural population of *Gloriosa superba* L., collected from Eastern Ghats of India for the identification of elite chemotypes
2.	Authors: Dr. Abhijit Dey *et al.* Name of Institute: Department of Life Sciences, Presidency University, Kolkata Title of Paper: Selection of elite germplasms for industrially viable medicinal crop *Bacopa monnieri* for bacoside A production: An HPTLC-coupled chemotaxonomic study
3.	Authors: Dr. Anandakumar Karunakaran *et al.* Name of Institute: Department of Pharmaceutical Analysis, Swamy Vivekanandha College of Pharmacy, Namakkal Title of Paper: Study of Various Factors impacting the Quality of *Terminalia Chebula* Fruit Rind Raw Material and Phytochemical Evaluation of Fruit, Fruit Rind and Nut Part of *Terminalia Chebula*

	Private Industry Winners
A	**1st Prize**
1.	Authors: Dr. Rahul Singh *et al.* Name of Company: R&D Center, Emami Ltd, Kolkata Title of Paper: Role of TLC to ensure manifestation of individual ingredients in herbal formulation
B	**Joint 2nd Prize**
1.	Authors: Dr. Aboli Girme *et al.* Name of Company: Pharmanza Herbal Pvt. Ltd., Anand Title of Paper: Assessment of *Curcuma longa* extract for adulteration with synthetic curcumin by analytical investigations
2.	Authors: Dr. Sulaiman CT *et al.* Name of Company: Phytochemistry Division, Centre for Medicinal Plants Research, Arya Vaidya Sala, Kottakkal Title of Paper: Identification of validated substitute for Asoka (*Saraca asoca* (Roxb.) Willd.) by phytochemical and pharmacological evaluations
C	**Joint 3rd Prize**
1.	Authors: Dr. Sharad Srivastava *et al.* Name of Institute: Pharmacognosy Division, CSIR National Botanical Research Institute, Lucknow Title of Paper: Simultaneous HPTLC-UV quantification of colchicine and gloriosine alkaloids in the natural population of *Gloriosa superba* L., collected from Eastern Ghats of India for the identification of elite chemotypes
2.	Authors: Dr. Anurag Varshney *et al.* Name of Institute: Drug Discovery and Development, Patanjali Research Institute, Haridwar Title of Paper: Tri-Herbal Medicine Divya Sarva-Kalp-Kwath (Livogrit) Regulates Fatty Acid-Induced Steatosis in Human HepG2 Cells through Inhibition of Intracellular Triglycerides and Extracellular Glycerol Levels

Screening for chlorinated paraffins in vegetable oils and dietary supplements by planar solid phase extraction



PD Dr. habil. Claudia Oellig and Prof. Dr. Michael Granvogl

The development of straightforward and simple screening methods for the analysis of chlorinated paraffins (CP) in vegetable oils and food with high fat content by planar solid phase extraction (pSPE), a technique that uses the performance and benefits of HPTLC, is one of the research topics of PD Dr. habil. Claudia Oellig at the Department of Food Chemistry and Analytical Chemistry (170a), directed by Prof. Dr. Michael Granvogl, Institute of Food Chemistry (University of Hohenheim, Stuttgart, Germany). This study shows a screening method for the determination of CP in vegetable oils and dietary supplements by pSPE and a comparison of the results obtained with a common gas chromatographic method.

Introduction

Chlorinated paraffins (CP) are complex mixtures of polychlorinated *n*-alkanes with 10 to 30 carbon atoms and variable chlorine contents (30–70%). CP are categorized by their carbon chain length as short- (SCCP; C_{10}–C_{13}), medium- (MCCP; C_{14}–C_{17}), and long-chain (LCCP; C_{18}–C_{30}) CP and they are widely used for technical applications, *e.g.*, they are added to plasticizers, paints, and flame retardants. CP are known to be persistent, and their bioaccumulation depends on the carbon chain length and the degree of chlorination. Because of their high toxicity, SCCP were classified as persistent organic pollutants (POP) according to the Stockholm Convention in 2017. MCCP and LCCP have similar chemical properties as SCCP, however, there have been only few studies about their toxicity and bioaccumulation, and their metabolism and distribution in the environment up to now, why they also pose a very high risk to humans. Due to their chemical structure, the degradation of CP in the environment is negligible, while accumulation in lipophilic tissues is very likely and high quantities might occur in lipids. CP were already detected in vegetable oils, milk, and dairy products, as well as in human milk fat and additionally in food supplements with high fat content. The analysis of CP is a very challenging task because CP are complex mixtures of polychlorinated *n*-alkanes, comprising thousands of congeners. The separation of these complex mixtures of unknown isomers and congeners is even not possible by gas chromatography (GC). Therefore, the presented screening method for CP uses the pSPE concept that quantifies CP as the sum. Based on the cost-effective and efficient HPTLC technique, pSPE guarantees reliable results for many samples simultaneously in a short time [1].

The determination of CP by applying the pSPE concept was successfully developed for a rapid and selective screening of CP as the sum. After sulfuric acid treatment and liquid-liquid partition into *n*-hexane, pSPE was performed on silica gel plates employing a twofold development. The analytes, which were focused in a single target zone, were determined by a densitometric absorption scan after derivatization with *o*-tolidine showing the total CP content, and the amounts were calculated as the sum by means of a reference CP. A comparison of the results obtained for the total CP in dietary supplements by pSPE with visual detection (pSPE–VIS) and by GC with high-resolution mass spectrometry (GC–HRMS) proved the pSPE approach as reliable tool for screening purposes.

Standard solutions

For method development, technical CP mixtures (SCCP with chlorine contents of 51.5%, 55.5%, and 63% and MCCP with chlorine contents of 42%, 52%, and 57%) are diluted in *n*-hexane to a concentration of 25 ng/µL. For the determination of the limit of decision and quantitation, recovery experiments in vegetable oils, and the analysis of dietary supplements, the reference CP (MCCP with a chlorine content of 52%) is diluted to concentrations of 0.075–0.8 ng/µL, 0.75–10 ng/µL, and 0.25–10 ng/µL, respectively. The internal standard (ISTD) 4,4-DDT is prepared at a concentration of 400 µg/mL (stock solution) and used at a final concentration of 6 ng/µL in all samples and standards. [1]

For GC–HRMS measurements, SCCP calibration standards with chlorine contents of 51.5%, 53.5%, 55.5%, 59.25%, and 63% and MCCP standards with chlorine contents of 42%, 47%, 52%, 54.5%, and 57% are prepared by respective dilutions of technical CP mixtures to a concentration of 10 ng/µL. The ISTD α-PDHCH and ε-HCH are added at concentrations of 0.075 and 0.05 ng/µL to all samples and standards. [1]

Sample preparation

Vegetable oil is treated according to Coelhan [2] with some modifications. After the addition of 15 µL of the ISTD 4,4-DDT stock solution to 250 mg of vegetable oil, 500 µL of *n*-hexane and 2.5 mL of concentrated sulfuric acid are added and the mixture is shaken for 3 min at 2200 rpm prior to centrifugation for 30 min. The *n*-hexane phase is separated, and the extraction is repeated with 500 µL of *n*-hexane under the same conditions. Both *n*-hexane extracts are combined and used for pSPE [1]. Oil-based dietary supplement capsules (250 mg of oil) are extracted by the same procedure as described above for the vegetable oil (following the procedure of the sulfuric acid treatment of the sample solution in *n*-hexane) including the addition of 7.5 µL of the ISTD α-PDHCH prior to the extraction (for GC–HRMS analysis). Furthermore, the combined *n*-hexane extracts (≈1 mL) are shaken with 2 mL of sulfuric acid for 4 min prior to centrifugation for 5 min. After separation of the *n*-hexane phase, the sulfuric acid treatment is repeated under the same conditions. A 300 µL aliquot of the *n*-hexane extract is used for pSPE and a 400 µL aliquot of the *n*-hexane extract including 2 µL of the ISTD ε-HCH is used for GC–HRMS. [1]

Chromatogram layer

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

Sample application

30 µL of samples and standards are applied as areas of 6 mm x 3 mm with the Automatic TLC Sampler (ATS 4), 22 tracks, distance from the left edge 13.0 mm and from the lower edge 8.0 mm. After application, plates are dried in a fume hood for 5 min.

Chromatography

Plates are developed twofold in the ADC 2 after activation at 33% relative humidity for 5 min using a saturated solution of magnesium chloride. The 1st development is performed with cyclohexane – toluene 94:6 (*V/V*) to a migration distance of 80 mm and the 2nd development is done with dichloromethane – *n*-hexane 90:10 (*V/V*) to a migration distance of 50 mm followed by a drying time of 3 min after each development.

Post-chromatographic derivatization

The plate is immersed into a solution of *o*-tolidine (4% in acetone) using the Chromatogram Immersion Device with an immersion speed of 3 cm/s and an immersion time of 1 s, dried for 4 min in a stream of cold air, and irradiated with UV-C light inside a self-made irradiation device using a cycle with irradiation and cooling steps [1].

Documentation

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

Densitometry

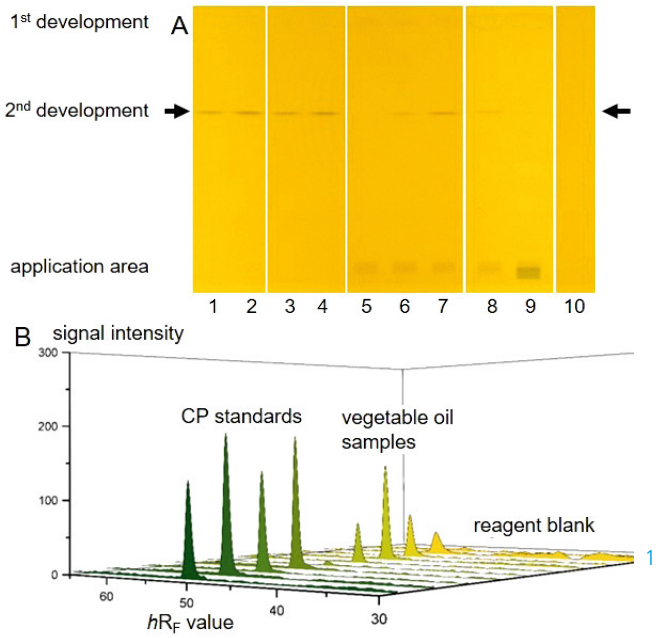
Absorbance measurement at 645 nm is performed with the TLC Scanner 4 with a scanning speed of 20 mm/s, a data resolution of 100 µm/step, and a slit dimension of 5 mm x 0.30 mm using the manual detector mode applying a quick scan range of 47–53 mm on the track of the most concentrated standard. For quantitation, the respective peak areas are used.

Gas chromatography coupled to high-resolution mass spectrometry

GC–HRMS measurements with electron capture negative ionization (ECNI) are performed according to the method of Krätschmer *et al.* [3] and quantitation is done according to Sprengel *et al.* [4] and Reth *et al.* [5].

Results and discussion

The determination of CP as the sum was performed by the straightforward pSPE approach. The target analytes were selectively separated from the matrix and additionally focused in a single and sharp target zone since all representatives of the chemical group of CP have almost identical properties in regard to a chromatographic separation. Best results were obtained by a twofold development on HPTLC silica gel with cyclohexane – toluene 94:6 (*V/V*) for the 1st development and dichloromethane – *n*-hexane 90:10 (*V/V*) for the 2nd development. In the target zone, the selective determination of the total CP as the sum was possible directly on the plate after derivatization with *o*-tolidine and UV irradiation by means of an absorption scan at 645 nm. For quantitation, a reference CP (MCCP with a chlorine content of 52%) was used.



(A) Plate image after pSPE and derivatization with o-tolidine in white light and (B) corresponding 3D densitogram of the absorption scan at 645 nm of (1–4) from left to right, SCCP (chlorine content 55.5% and 63%), MCCP (chlorine content 52% and 57%), 225 ng CP/zone; (5–9) different vegetable oils, 7.5 mg sample/zone; (10) a reagent blank.

Limits of decision and quantitation for the reference CP were determined according to the DIN 32645 calibration method [6] in the range of 2.25–24 ng CP/zone (corresponding to 0.3–3.2 mg CP/kg of vegetable oil) and 1.5–4.9 ng CP/zone (corresponding to 0.2–0.7 mg CP/kg of vegetable oil), respectively, with relative standard deviations (RSD) <5%. Thus, the presented pSPE–VIS approach delivers a suitable screening tool for the analysis of the total CP down to ~1 mg/kg of vegetable oil, while for lower limits, sample application volume can be increased.

Recoveries for CP in sunflower oil, olive oil, and rice bran oil at levels of 5, 10, and 30 mg reference CP/kg were very close to 100% after the subtraction of a slight impurity originating from the sample preparation. With precision of recovery expressed as RSD <4%, the method was very well repeatable for all tested vegetable oils and spiking levels.

Mean recoveries of CP from vegetable oils in % ± standard deviation ($n = 4$) at spiking levels of 5, 10, and 30 mg reference CP/kg of oil. Modified from [1].

	Spiking level[a]		
Vegetable oil	5 mg/kg	10 mg/kg	30 mg/kg
1	97 ± 2	99 ± 2	92 ± 1
2	98 ± 2	102 ± 3	97 ± 4
3	99 ± 1	102 ± 2	98 ± 2

[a] Spiked with the reference CP and quantified against the reference CP.

Comparison of results obtained by pSPE–VIS and GC–HRMS for six dietary supplement samples (oil-based food supplements) showed generally conformity. The total CP contents determined by pSPE–VIS were in the same order of magnitude compared to the contents analyzed by GC–HRMS and results were well repeatable. Only one sample (sample 3) showed a clearly higher content by pSPE, however, displayed a different color of the target zone that evidently originated from co-migrated matrix components. In general, large deviations in results are well known for the very challenging CP analysis, which depend on the used GC–MS set-up including the calibration standards and the calibration method.

Total CP amounts in six dietary supplement samples from the European market by pSPE–VIS and GC/ECNI–HRMS in mg reference CP/kg of sample ± standard deviation ($n = 4$). Modified from [1].

Dietary supplement sample	pSPE–VIS[a]	GC/ECNI–HRMS[b]
1	18.9 ± 0.4	6.9 ± 0.4
2	15.3 ± 0.1	10.2 ± 0.2
3	4.9 ± 0.2	0.6 ± 0.1
4	<limit of quantitation	–
5	28.5 ± 0.5	14.9 ± 0.2
6	~limit of quantitation	–

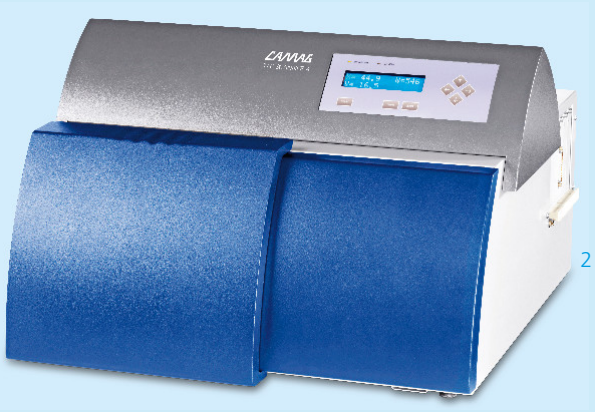
[a] Quantified against the reference CP and expressed as mg reference CP/kg of sample.
[b] Quantified against the technical SCCP and MCCP mixtures according to [4], summed up, and expressed as mg CP/kg of sample.

In summary, the pSPE screening approach for the determination of the total CP provides a very useful alternative to the time-consuming and very complicated GC approaches that analyze the individual components and calculate the sum afterwards. Furthermore, in terms of a rapid screening, pSPE offers the chance to save time and costs, because only the suspected and questionable samples need additionally to be analyzed by GC/ECNI–HRMS.

[1] C. Oellig *et al.*, J Chromatogr A (2019) 460380.
[2] M. Coelhan, Anal Chem (1999) 4498.
[3] K. Krätschmer *et al.*, J Chromatogr A (2018) 53.
[4] J. Sprengel *et al.*, Rapid Commun Mass Spectrom (2018) 49.
[5] M. Reth *et al.*, J Chromatogr A (2005) 225.
[6] Deutsches Institut für Normung, DIN 32645, Beuth, Berlin, 2008.

Further information is available on request from the authors.

Contact: PD Dr. habil. Claudia Oellig, Department of Food Chemistry and Analytical Chemistry (170a), Institute of Food Chemistry, University of Hohenheim, 70599 Stuttgart, claudia.oellig@uni-hohenheim.de



2

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The TLC Scanner 4 is the most advanced workstation for densitometric evaluation of TLC/HPTLC chromatograms. It measures the reflection of separated compounds in absorbance and/or fluorescence mode.
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Identification and quantification of glucose degradation products

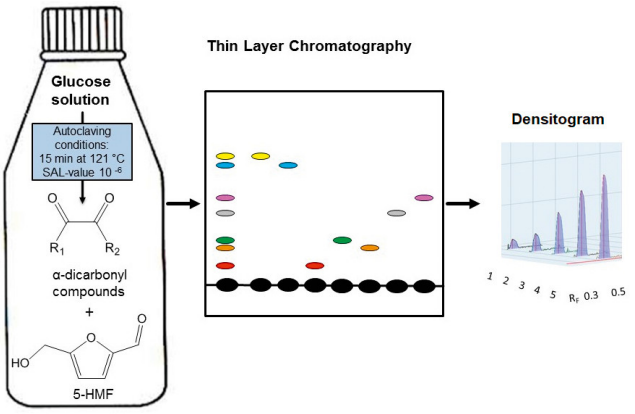
Sarah Leitzen

The subject of this article was investigated by Prof. Dr. Martin Brandl, Anette Engels, Dr. Matthias Vogel, Dr. Thomas Zapf and Sarah Leitzen [1]. Prof. Dr. Martin Brandl is a pharmacist and professor at the Department of Physics, Chemistry and Pharmacy at the University of Southern Denmark (SDU), Odense, Denmark and supervisor of Sarah Leitzen's doctoral thesis. Anette Engels, Dr. Matthias Vogel and Dr. Thomas Zapf work at the Federal Institute for Drugs and Medical Devices (BfArM) in Bonn. Sarah Leitzen is a pharmacist and PhD student working at both BfArM and SDU.

Introduction

Sterile glucose solutions are commonly used as reconstitution solvents or diluents for injectable drugs and also for peritoneal dialysis solutions. In Germany, regulatory requirements for the different strengths of glucose solutions used for parenteral administration are regulated and published as standard marketing authorizations.

During heat sterilization of glucose solutions for parenteral use, a variety of glucose degradation products (GDPs) may be formed. GDPs can cause cytotoxic effects after parenteral administration of these solutions. Therefore, the aim of the current study was to develop a simple and quick HPTLC method by which the major GDPs can be identified and (summarily) quantified in glucose solutions for parenteral administration. All GDPs were derivatized with *o*-phenylenediamine (OPD). The identity of the resulting GDP derivatives (quinoxalines) during method validation was confirmed via mass spectrometry



1

Graphical abstract from [1] (https://creativecommons.org/licenses/by/4.0/legalcode).

Standard solutions and pre-chromatographic derivatization

For each degradation product (glyoxal (GO), methylglyoxal (MGO), glucosone (2-KDG), 3-deoxyglucosone (3-DG), 3-deoxygalactosone (3-DGal), 3,4-dideoxyglucosone-3-ene (3,4-DGE), and the impurity 5-hydroxymethylfurfural (5-HMF)), individual 0.5 mg/mL solutions are prepared with fresh ultrapure water, as well as a mix of all seven GDPs. For quantification, calibration standards containing all seven GDPs at concentration levels of 1–75 µg/mL are prepared. All solutions also contain 50 mg/mL glucose and 0.75 mg/mL OPD. They are left to stand in the dark for 16 hours at room temperature.

Sample preparation and pre-chromatographic derivatization

An artificial mix is prepared to simulate expected concentrations of GDPs in an autoclaved 5% glucose solution containing GO and MGO (1.0 mg/mL, each), 2-KDG (7.0 mg/mL), 3-DG (45.0 mg/mL), 3-DGal (25.0 mg/mL), 3,4-DGE and 5-HMF (5.0 mg/mL, each). The mix also contains glucose in a concentration of 50 mg/mL and OPD as derivatization reagent in a concentration of 0.75 mg/mL.

Samples are analyzed after a 16 h reaction time at room temperature.

Chromatogram layer

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

Sample application

Samples and standard solutions are applied as bands with the Automatic TLC Sampler (ATS 4), band length 10.0 mm, distance between tracks of 19.0 mm, distance from lower edge 29.0 mm. 10 µL for sample and standard solutions are applied.

Chromatography

Plates are developed in the Twin Trough Chamber (TTC 20 x 20 cm) with chamber saturation (with filter paper) for 20 min, development with 25 mL of 1,4-dioxane – toluene – glacial acetic acid 49:49:2 (*V/V*) (each trough) to the migration distance of 160 mm (from the lower edge), followed by drying for 10 min.

Editor's note: in this special case, a migration distance of 160 mm leads to a significantly improved separation on HPTLC with the selected solvent system. Usually, a migration exceeding 80 mm on HPTLC plates does not improve resolution due to increasing diffusion

Post-chromatographic derivatization (second derivatization)

The plates are sprayed with the Derivatizer (yellow nozzle, level 4, 2 mL) with thymol-sulfuric acid reagent (2 mL of a solution of 0.5 g of thymol in a mixture of 5 mL of sulfuric acid and 95 mL of ethanol 96%) and heated at 130 °C for 10 minutes on the TLC Plate Heater.

Authors' Note: While establishing the method, two identical plates were run simultaneously (in separate twin trough chambers with chamber saturation), where one of the two plates was not treated with thymol-sulfuric acid reagent for substance confirmation by mass spectrometry.

Documentation

Images of the plate are captured with the TLC Visualizer in UV 254 nm, UV 366 nm, and white light.

Densitometry

The HPTLC plate was scanned in absorbance mode at 330, 370, and 420 nm, and in fluorescence mode at 366>/400 nm.

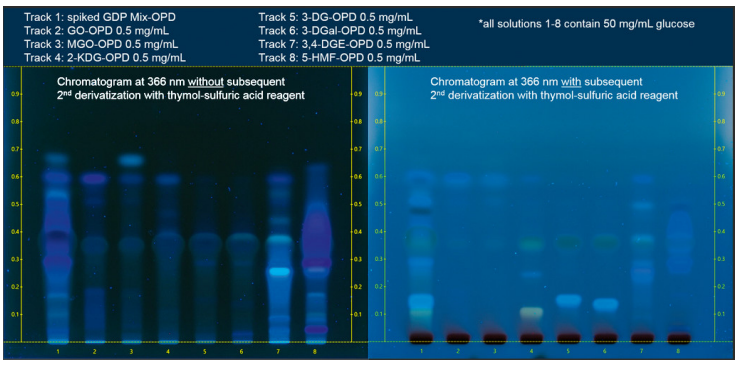
Mass spectrometry

One of the duplicate plates (plate without 2nd derivatization step) is used for substance confirmation. The zones are localized with the help of the plate derivatized with thymol-sulfuric acid reagent. Zones of interest are scraped off at the expected position and extracted three times, using 200 µL of methanol each time, and centrifuged for 2 min at 16,000 rpm/21,130 rcf. The supernatants are evaporated with nitrogen and the residues are finally reconstituted in 200 µL of a 5 mM ammonium acetate buffer solution (pH = 3.5). The solutions are analyzed in positive electrospray ionization mode [1].

Editor's Note: The zones can also directly be eluted to an MS for substance confirmation with the TLC-MS Interface 2. However, this equipment was not available at the BfArM.

Results and discussion

Glucose, as a highly polar molecule, does not react with OPD, or only to a lesser degree, and maintains its hydrophilic character without forming the quinoxaline system. Therefore, glucose is observed at the application position.



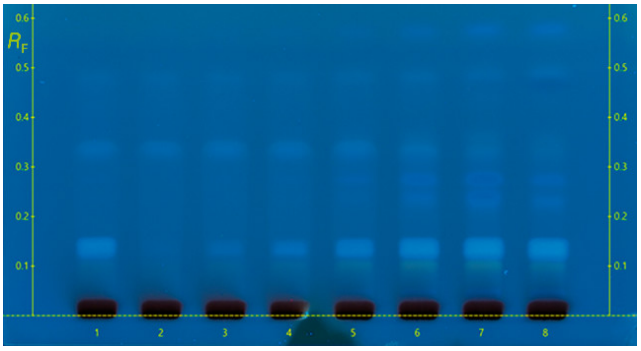
2

Chromatograms of the derivatized seven GDPs and a mixture (= spiked glucose solution) in the solvent 1,4-dioxane – toluene – glacial acetic acid 49:49:2 (V/V). The chromatograms are shown in UV 366 nm without (left) and with (right) subsequent 2nd derivatization with thymol-sulfuric acid reagent at concentration levels of 0.5 mg/mL [1]. (https://creativecommons.org/licenses/by/4.0/legalcode).

Overview of the obtained R_F values and signal responses of each analyte at different wavelengths *without and **with second derivatization.

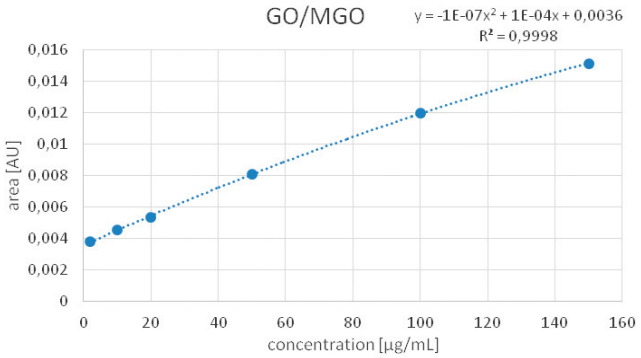
Analyte	R_F	*UV 254 nm	*UV 366 nm	*UV 254 nm	*UV 366 nm
Glucose	0.00	–	–	0	++
2-KDG	0.10	+	–	++	++
3-DG	0.12	+	–	++	++
3-DGal	0.14	+	–	++	++
3,4-DGE	0.24	+	++	0	+
5-HMF	0.28	+	++	0	+
GO	0.57	+	+	+	++
MGO	0.57	++	+	+	++

++ Very well visible, + Clearly visible, 0 Faintly visible, – Cannot be seen



3

HPTLC chromatograms in UV 366 nm after both derivatizations (ROI until R_F 0.6). Track 1 shows the spiked 5% glucose solution spiked GDP-Mix-OPD); tracks 2–8 the calibration solutions in ascending concentrations (1.0 µg/mL–100.0 µg/mL GDPs) [1]. All solutions contain 50 mg/mL glucose. (https://creativecommons.org/licenses/by/4.0/legalcode).



4

Calibration curve of GO/MGO at concentrations of 2–150 µg/mL (measured in absorbance mode at 330 nm) [1]. (https://creativecommons.org/licenses/by/4.0/legalcode).

The presented method was successfully validated using the ICH Q2(R1) guideline. For 2-KDG, the linearity of the method was demonstrated in the range of 1–50 µg/mL, for 5-HMF and 3,4-DGE 1–75 µg/mL, for GO/MGO 2–150 µg/mL, and for 3-DG/3-DGal 10–150 µg/mL. All GDPs achieved a limit of detection (LOD) of 2 µg/mL or less and a limit of quantification (LOQ) of 10 µg/mL or less. R^2 was 0.982 for 3.4-DGE, 0.997 for 5-HMF, and 0.999 for 2-KDG, 3-DG/3-DGal, and GO/MGO. The intraday precision was between 0.4 and 14.2% and the accuracy, reported as % recovery, between 86.4 and 112.7%. The proposed HPTLC method appears to be an inexpensive, fast, and sufficiently sensitive approach for routine quantitative analysis of GDPs in heat-sterilized glucose solutions.

[1] Leitzen *et al.* PLoS ONE (2021), https://doi.org/10.1371/journal.pone.0253811

Further information is available on request from the author.

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5

HPTLC Online User Meetings, hosted by the international HPTLC Association

To facilitate the worldwide knowledge exchange between HPTLC users, the series of HPTLC Online User Meetings was created.

The first HPTLC Online User Meeting was organized by the North America Chapter of the HPTLC Association. Held on 17 March 2022, it featured three exciting presentations on herbal drug and forensic applications and attracted more than 1,000 HPTLC users worldwide. In a separate session immediately after the main event, attendees engaged in moderated discussions with speakers, colleagues, and representatives from the HPTLC Association. To view the presentations on demand, visit **https://bit.ly/37AKJSH**.

The second HPTLC Online User Meeting is organized by the Germany Chapter and will be held on 16 June 2022.

The HPTLC Association Substance Database: a useful tool for identification of compounds.

Dr. Thi Kieu Tiên Do, Dr. René De Vaumas, Dr. Eike Reich

The idea of developing a substance database for natural products arose from a previous collaboration on HPTLC of flavonoids and phenolic acids, between CAMAG and the ZHAW (Zurich University of Applied Sciences, Wädenswil). The project investigated the chromatographic behavior of about 72 substances. In a pilot phase in collaboration with the company Extrasynthese we worked on two smaller projects on iridoids and coumarins to practically implement the database concept.

Introduction

The HPTLC Association Substance Database is a systematic collection of data, easily accessible electronically from the website of the HPTLC Association [1]. It contains retention and spectral data of representative substances from different classes, analyzed with different developing solvents and derivatization reagents.

With the substance database, HPTLC laboratories have a free and convenient tool to help with identification of zones in a chromatogram. The user can compare R_F values, and colors prior to and after derivatization, and UV spectra of unknown zones with those of the references. As the collection will be regularly expanded with substances not limited to constituents of herbal drugs, different fields of application can benefit from the HPTLC Association substance database. Iridoids and coumarins are described in this article.

Standard solutions

Iridoids and coumarins were dissolved in methanol at a concentration of 1.0 mg/mL, and concentrations were adjusted if needed.

Chromatogram layer

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

Sample application

2.0 µL of solutions were 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 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. Different mobile phases were investigated (see table), followed by drying for 5 min.

Post-chromatographic derivatization

Iridoids were derivatized with anisaldehyde sulfuric acid (AS) and vanillin sulfuric acid (VS) reagents. Coumarins were derivatized with AS, potassium hydroxide (KOH), and natural product (NP) / polyethylene glycol (PEG) reagents.

AS reagent: slowly and carefully mix 170.0 mL of ice-cooled methanol with 20.0 mL of acetic acid and 10.0 mL of sulfuric acid; allow the mixture to cool to room temperature, then add 1.0 mL of anisaldehyde (p-methoxy benzaldehyde); VS: dissolve 1 g of vanillin in 100 mL of ethanol 96% and carefully add 2 mL of concentrated sulfuric acid; KOH: dissolve 5 g potassium hydroxide in 100 mL of methanol (96%); NP: dissolve 1.0 g of 2-aminoethyl diphenylborinate in 200 mL of ethyl acetate; PEG: mix 10 g of polyethylene glycol 400 (macrogol) with 200 mL of dichloromethane.

Documentation

Images of the plates were captured with the TLC Visualizer 2 in UV 254 nm, UV 366 nm and white light, and in UV 366 nm and white light after derivatization.

Densitometry

Absorbance measurements at 254 nm with the deuterium lamp for iridoids, and at 313 nm with the mercury lamp for coumarins were performed with TLC Scanner 4 and *visionCATS*, slit dimension 5.00 mm x 0.20 mm, scanning speed 20 mm/s. Spectra of the corresponding zones were recorded in the absorbance-reflectance mode from λ= 190–450 nm using a combination of deuterium and tungsten lamps with a slit size of 5.00 mm x 0.20 mm.

Results and discussion

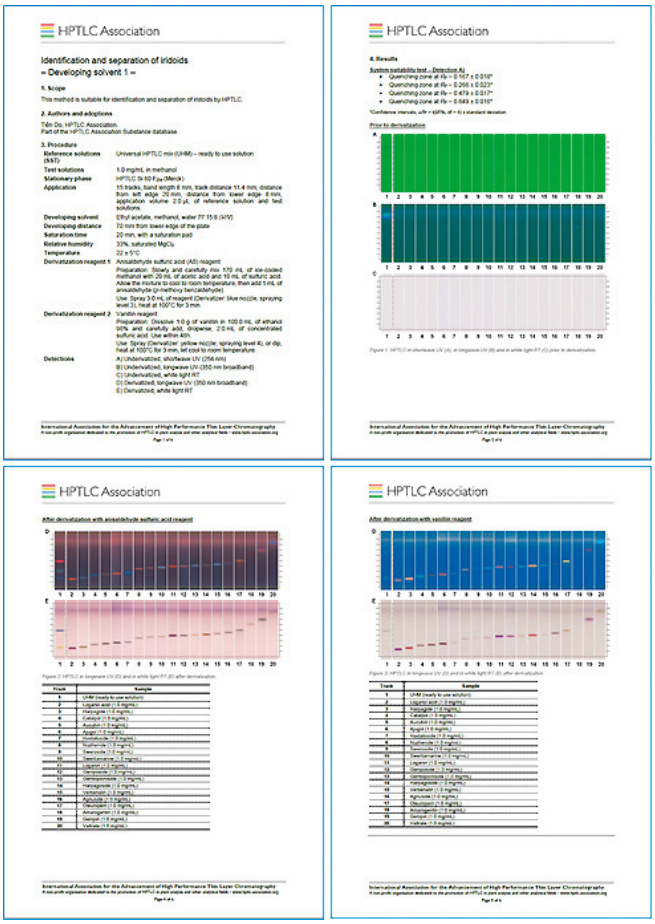
Analysts who want to identify unknown zones in the HPTLC chromatogram are limited to the availability of reference standards in their laboratory. To help with selecting appropriate standards, the substance database can be a useful screening tool.

For each class of compounds, six steps were taken: evaluation of the existing HPTLC methods, selection of three developing solvents of diverse properties, evaluation of the derivatization reagents, analysis of all substances of each class, and creation of the documentation.

		Mobile phase	Polarity index	Selectivity groups	Derivatization reagent
Iridoids	A	Ethyl acetate, methanol, water 77:15:8 (*V/V*)	4.97	VIa, II, VIII	AS, VS
	B	Ethanol, dichloromethane, water 45:70:6.5 (*V/V*)	18.26	II, V, VIII	AS, VS
	C	Ethyl acetate, formic acid, acetic acid, water 100:11:11:26 (*V/V*)	8.47	VIa, IV, IV, VIII	AS, VS
Coumarins	D	Dichloromethane, heptane, ethyl acetate 4:4:2 (*V/V*)	2.16	V, VIa	AS, KOH, NP/PEG, sulfuric acid
	E	Toluene, diethyl ether 1:1 (*V/V*)	2.60	VII, I	AS, KOH, NP/PEG, sulfuric acid
	F	Toluene, ethyl acetate 90:10 (*V/V*)	2.60	VII, VIa	AS, KOH, NP/PEG, sulfuric acid

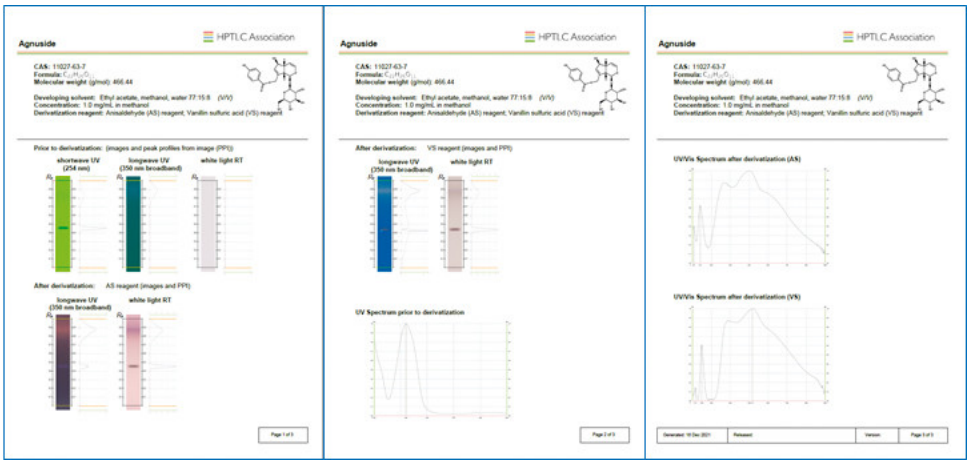
[Note]: Heptane is not included in selectivity groups. AS: anisaldehyde sulfuric acid. VS: vanillin sulfuric acid, KOH: potassium hydroxide; NP: natural product; PEG: polyethylene glycol.

The HPTLC Association provides method documents (PDF format) for each class and all evaluated developing solvents for a convenient check of available substances.



Method document for iridoids, developing solvent A [1]

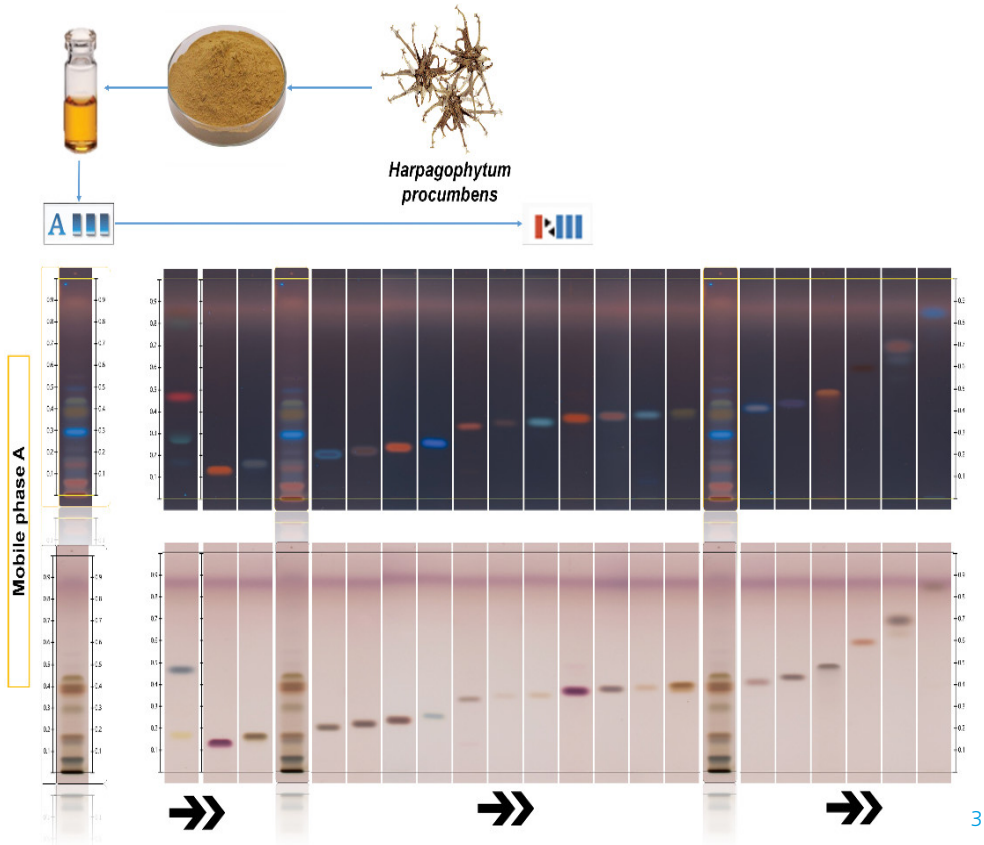
The documentation of the database also includes PDF files for each individual substance including peak profiles from image, UV spectra and images of chromatograms prior to and after derivatization.



2

Substance document of agnuside, developing solvent A

Users of *visionCATS* can download all the data as comparison files and import their own data for comparison.



3

Use of the comparison file from the substance database for establishing the presence of harpagoside and harpagide in Harpagophytum procumbens.

[1] T. K. T. Do, R. De Vaumas, E. Reich. J Planar Chromatogr 1638 (2021).
DOI: 10.1016/j.chroma.2020.461830.

Further information is available on request from the authors.

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HPTLC detection of falsification in drugs

From left: Carole Vrignaud (Control Laboratory Manager), Baptiste Ragueneau (Temporary Analyst) and Patricia Morel (Analyst)

Mrs. Carole Vrignaud and her colleagues at the Anti-counterfeiting Laboratory (LCAC) at Sanofi Tours, France, employ chromatographic separation techniques, especially HPTLC, to develop new qualitative analytical methods for detection of falsified drugs, in a variety of sample matrices. The world drug market is about 1000 billion € (2019). The World Health Organization (WHO) considers that half of the drugs sold through the internet are fakes. This is why Sanofi group in 2008 created this unique laboratory to fight against drug falsification using various analytical methods, including HPTLC. The main objective is to confirm the presence of active ingredients (API) or excipients (preservatives, flavoring…) in each Sanofi formulation. HPTLC is also often used to detect degradation of products stored under inappropriate conditions, as well as unexpected compounds, allowing characterization of the falsified products. To detect falsification of drugs, a generic HPTLC method was developed.

HPTLC is well suited for a rapid parallel screening of many samples. Up to 10 samples can be analyzed in less than one hour. The developed method is simple and can be performed with little consumption of solvents and reagents.

Standard solutions

20 mg of API no. 1, 55 mg of API no. 2, 53 mg of API no. 3, and 51 mg of impurity of API no. 2 are individually dissolved in 10 mL of water (each).

Sample preparation

One tablet of each suspected case of falsification is milled, and then extracted with 7.5 mL of water by vortexing. After centrifugation, the filtered supernatant is transferred into a 5 mL volumetric flask and filled up to the mark.

Chromatogram layer

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

Sample application

Samples and standard solutions are applied as bands with the Automatic TLC Sampler (ATS 4), 14 tracks, band length 8.0 mm, distance from left edge 20.0 mm, distance from lower edge 8.0 mm. 1.0 µL of sample solutions and 3.0 µL of standard solutions are applied.

Chromatography

In the ADC 2 with chamber saturation (20 min) and after conditioning at 33% relative humidity for 10 min using a saturated solution of magnesium chloride, development with dichloromethane – ethanol – water – formic acid 9:9:1.5:0.25 (*V/V*) to the migration distance of 50 mm (from the lower edge), followed by drying for 5 min.

Post-chromatographic derivatization

First, the plates are manually sprayed with Dragendorff's reagent (commercial solution) and dried for 60 s at room temperature. Second, the plates are manually sprayed with sodium nitrite solution (5%) and dried for 30 s at room temperature.

Documentation

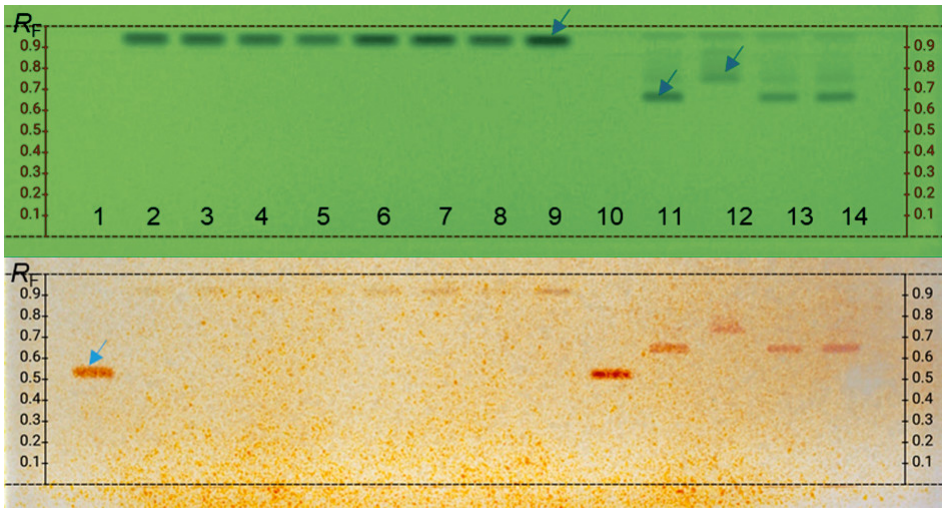
Images of the plates are captured with the TLC Visualizer in UV 254 nm before derivatization and in white light after each derivatization step.

Densitometry

Absorbance measurement is performed with the TLC Scanner 3 and *visionCATS* at 272 nm, slit dimension 5.0 mm × 0.2 mm, measurement speed 20 mm/s, spectra recording from 200 to 450 nm.

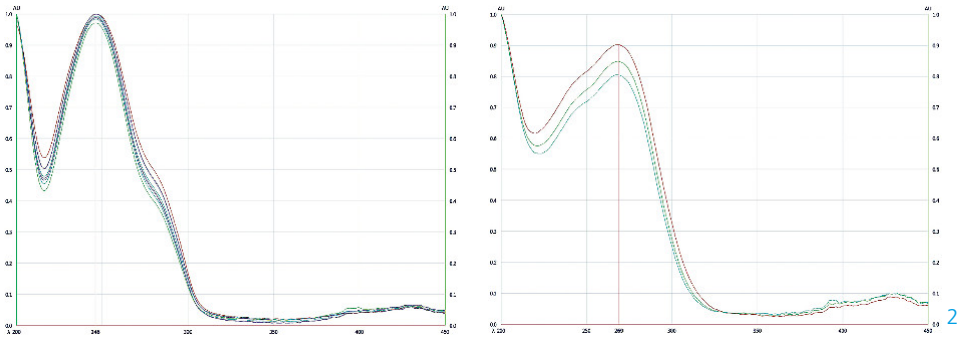
Results and discussion

A representative plate with real samples is shown. During method development simulated and spiked samples gave the same R_F values as the standards and were well separated from matrix components. Any positive identification of API no. 2 and no. 3 can be confirmed by spectral comparison of samples and standards as well as the expected presence of impurity of API no. 2. Detection of API no. 1 (cases no. 1–7) is achieved after derivatization in white light. Results are shown for selected drug products as well as the identification of three active ingredients expected in different oral formulations. HPTLC is also used to estimate each quantity. API no. 3 is not present in the composition of the original tablets (cases no. 8–9). However, some previous cases were identified as counterfeit tablets containing API no. 3 instead of API no. 2.



1

HPTLC chromatogram (top) in UV 254 nm prior to and (bottom) in white light after derivatization; tracks 1&10: API no. 1; tracks 2–8: cases no. 1–7; track 9: API no. 3; track 11: API no. 2; track 12: impurity of API no. 2; tracks 13–14: cases no. 8–9



2

(Left) UV spectrum API no. 3 (red) and samples (cases no. 1-7); (right) UV spectrum API no. 2 (red) and samples (cases no. 8–9)

In the shown real cases no falsification was detected. Nevertheless, the approach has proved suitable by several positive cases. HPTLC is therefore used at Sanofi as high throughput screening technique.

Further information is available on request from the author.

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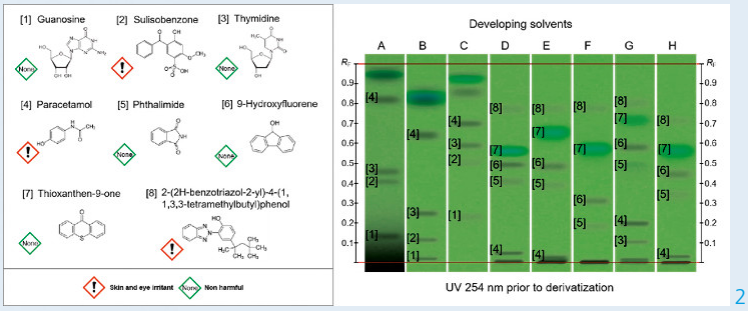
carole.vrignaud@sanofi.com

Universal HPTLC Mix (UHM)

1

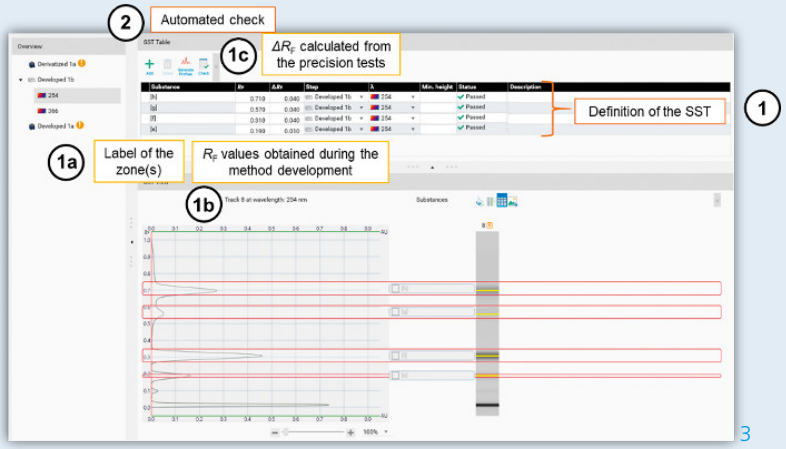
UHM in practice by using CAMAG's visionCATS HPTLC software

Ready to use analytical standard solution for HPTLC system suitability tests (SST)



2

Left: Substances selected for UHM. Right: HPTLC fingerprints of the UHM in eight different developing solvents [2]



3

The unique Universal HPTLC Mix (UHM) provides HPTLC laboratories with a single solution for system suitability tests (SST) of a wide range of chromatographic systems with different polarities and selectivities. Replacing conventional SST substances by the UHM helps laboratories save time and money – no more laborious investigations of specific reference substances for the qualification of analytical methods.

Key benefits:

- One single solution for SST
- Lower costs
- Stability in solution
- Capability to detect small chromatographic variations

The suitability of the UHM as universal SST in selected pharmacopeia monographs for the identification of herbal drugs was demonstrated in a recent publication [1].

Developed in collaboration with CAMAG, the UHM (HPTLC calibration mix 91816) can be purchased from *Merck KGaA.

www.sigmaaldrich.com/uhm.

[1] M. Schmid *et al.* (2022) J Chromatogr A, 1666. DOI: 10.1016/j.chroma.2022.462863

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

*The life science business of Merck KGaA, Darmstadt, Germany operates as MilliporeSigma in the U.S. and Canada.

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