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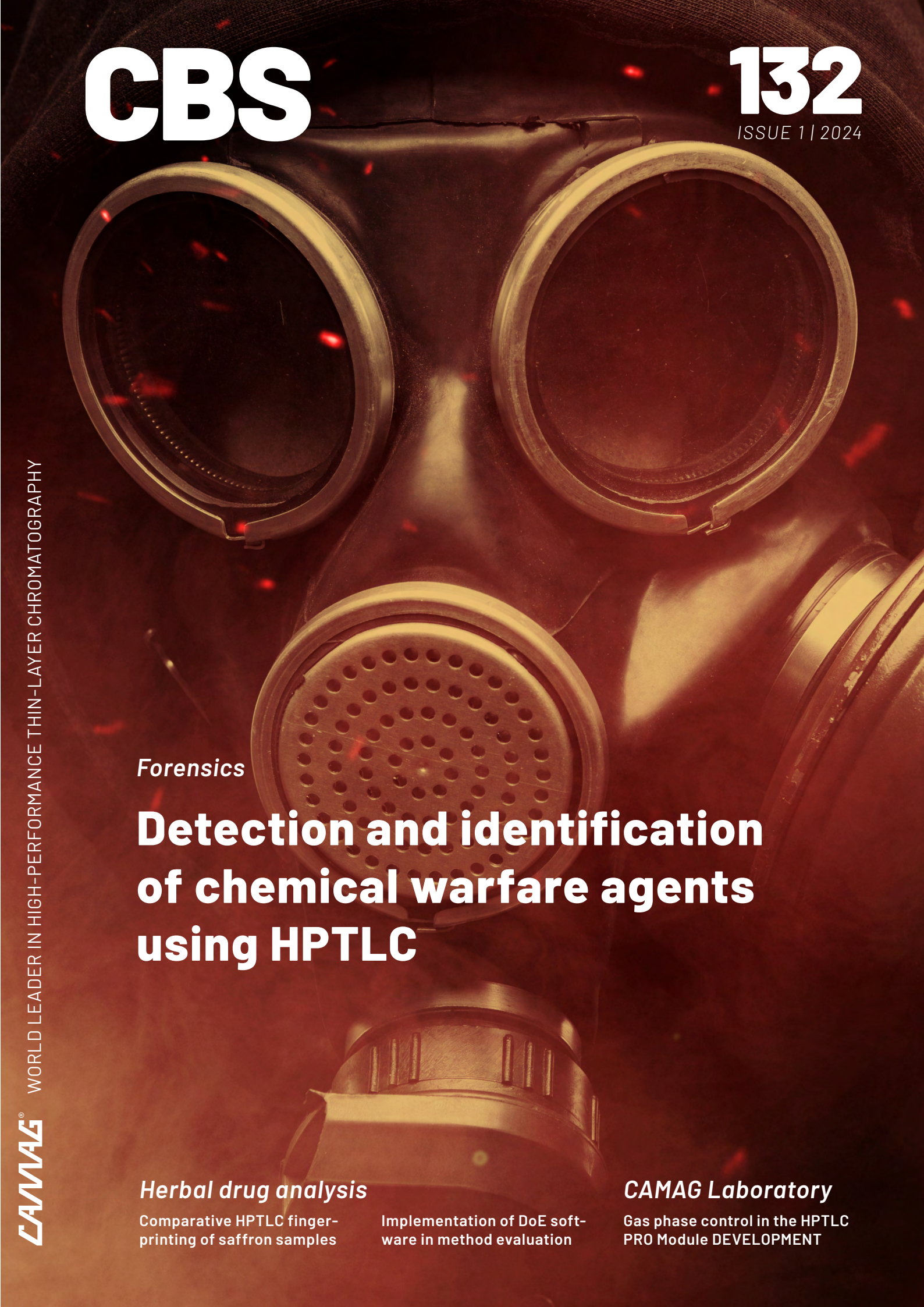
ISSUE 1 | 2024



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Forensics

Detection and identification of chemical warfare agents using HPTLC

Herbal drug analysis

Comparative HPTLC fingerprinting of saffron samples

Implementation of DoE software in method evaluation

CAMAG Laboratory

Gas phase control in the HPTLC PRO Module DEVELOPMENT

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No. 132, Issue 1 | 2024

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Procedures, applications

CAMAG (Switzerland)
Sonnenmattstrasse 11 | 4132 Muttenz
info@camag.com | www.camag.com

Note from the editor

Dear friends of HPTLC,

Welcome to the latest issue of the CBS Journal. We are pleased to present you with a variety of articles highlighting advances in the field of HPTLC.

First, we delve into the critical area of chemical warfare agent detection. Through the lens of HPTLC, we learn about a powerful screening method to identify nerve agents, blistering agents and irritants, as well as arsenic agents. This work is presented by the Swiss Army and experts from CAMAG.

Our focus then shifts to the quality assessment of saffron, a valuable commodity in both medicine and food. This successful work is presented by the Anchrom laboratory in India.

We then discover the implementation of Design of Experiments (DoE) software in method evaluation using a compendial method. This approach, demonstrated by the company Wala and the Esslingen University of Applied Sciences in Germany, aims to streamline robustness studies and provide pharmaceutical manufacturers with efficient tools for method validation and compliance.

Finally, we turn our attention to the realm of separation with a study that explores the manipulation of the gas phase. The CAMAG laboratory examines the features of the HPTLC PRO Module DEVELOPMENT with the aim of refining chromatographic separations through gas phase control.

We hope you find this edition as enlightening and informative as we have in assembling it. As always, we welcome your feedback and look forward to continuing this journey of exploration and discovery together.

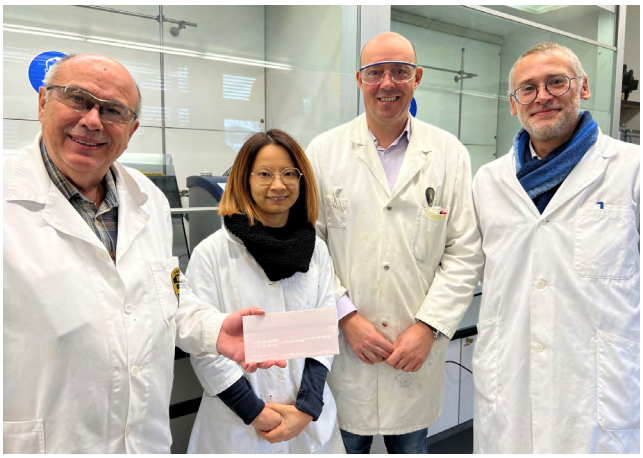
Yours sincerely,

Prof. Dr. Melanie Broszat

Editor CBS

Detection and identification of chemical warfare agents using HPTLC

Patrick Bargsten (Swiss Army), Tiên Do and Raphaël Vizzini (CAMAG), and Beat Schnyder (Swiss Army)



HPTLC is a reliable and widely used analytical technique for the identification of chemical warfare. HPTLC separates the individual components of a mixture, making it possible to identify specific nerve agents such as Russian VX (RVX), O-ethyl S-(2-diisopropylaminoethyl) methylphosphonothioate (VX), Soman (GD), Tabun (GA), cyclosarin (GF), and sarin (GB) based on their characteristic retention factor (R_F) values [1]. TLC methods were transferred to HPTLC.

For six blistering agents and irritants, namely sulfur mustard (HD), HN-3 (TTA), 2-chlorobenzylidenemalononitrile (CS), 2-chloroacetophenone (CN), bromobenzyl cyanide (CA), and benzyl bromide (CB) [2], as well as three arsenic agents Lewisite (L), Clark 1 (DA), and Adamsite (DM) [3], their initial TLC methods were successfully transferred to HPTLC. This underscores the adaptability and efficacy of HPTLC in extending the capabilities of traditional TLC methods for the comprehensive analysis of chemical warfare agents.

Introduction

Chemical warfare agents present a considerable threat to human health, inducing a spectrum of symptoms ranging from irritation to fatality. It is imperative for law enforcement agencies and military personnel to possess the knowledge and tools required to detect and prevent exposure to these hazardous substances. There are various methods to categorize chemical warfare agents, one common approach is to categorize them based on the primary symptoms they cause. Nerve agents, for instance, are organic chemicals that disrupt the mechanisms through which nerves convey messages to organs. This disruption arises from the inhibition of acetylcholinesterase (AChE), an enzyme facilitating the breakdown of acetylcholine.

Blistering agents, also known as vesicants, are chemical warfare agents that induce skin blisters, eye damage, and respiratory harm. Typically, these agents manifest as oily liquids that can persist on surfaces for extended durations. Exposure to blistering agents can lead to severe burns, lung damage, and even death. In contrast, irritant agents elicit irritation on the skin, eyes, and respiratory system. Although less lethal than nerve agents and blistering agents, irritant agents can still inflict significant harm on exposed individuals. Examples of irritant agents include substances like chlorine gas, phosgene gas, and tear gas.

Arsenic agents represent another category of chemical warfare agents capable of causing substantial harm to human health. Exposure to arsenic agents can result in symptoms ranging from irritation to death.

Standard solutions

Individual standard solutions were prepared according to the table below, and for quantification purposes each solution was applied at different application volumes to generate a calibration curve.

System Suitability Test (SST): the ready-to-use solution of Universal HPTLC mix (UHM) was prepared in house according to [4] and applied on track 8 of each plate.

Nerve agents	GA, VX, RVX were prepared at 0.5 ng/µL, GB at 0.25 ng/µL, GD at 0.05 ng/µL, and GF at 0.02 ng/µL in dichloromethane
Blistering agents and irritants	TTA and CB were prepared at 5.0 µg/µL, HD at 2.5 µg/µL, CA at 0.5 µg/µL, and CS and CN were prepared at 0.25 µg/µL in dichloromethane
Arsenic agents	DM and DA were prepared at 0.25 µg/µL, and L at 1.25 µg/µL in dichloromethane

Chromatogram layer

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

Sample application

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

Chromatography

Plates were developed with the following three developing solvents in ADC 2 with activation of the plate at 33 % relative humidity for 10 min using a saturated solution of magnesium chloride.

For nerve agents, acetone – cyclohexane – ethyl acetate – methanol 1:5:3:0.2 (*V/V*), for blistering agents and irritants, toluene, and for arsenic agents, cyclohexane – dichloromethane – methanol 7:2:1 (*V/V*), are used as developing solvents with 20 min chamber saturation (with saturation pad). The developing distance for all three methods was 70 mm (from the lower edge). Plates were dried for 5 min.

Post-chromatographic derivatization

For nerve agents:

1. Spraying solution A: Acetylcholinesterase

Reagent preparation:
Dissolve 55.0 mg of acetylcholinesterase (55 mg = 150 U) in 100.0 mL of buffer solution (dissolve 19.0 g of Na_2HPO_4 x 12 H_2O and 1.8 g of KH_2PO_4 in 1.0 L of de-ionized water (pH approx. 7.4)).

Reagent use:
Spray the plate with 4.0 mL of spraying solution A with the Derivatizer, yellow nozzle, spraying level 4, and leave the plate (horizontal; outside of the Derivatizer) for 15 min at room temperature. [Note]: with 4.0 mL, the plate should not dry out.

2. Spraying solution B: Fast blue salt

Reagent preparation:
Mix 40.0 mL of fast blue solution (100.0 mg of fast blue salt B in 40.0 mL of de-ionized water) with 10.0 mL of 1-naphthyl acetate solution (25.0 mg of 1-naphthylacetate in 10.0 mL of ethanol).

Reagent use:
Spray the plate with 2.0 mL of praying solution B with the Derivatizer, yellow nozzle, spraying level 4, and record the images after 30 min.

For blistering agents and irritants (optional):

1. Spraying solution C: 4-(4'-Nitroenzyl)-pyridine solution

Reagent preparation:
Dissolve 5.0 g of 4-(4'-nitrobenzyl)-pyridine in 100.0 mL of ethanol.

2. Spraying solution D: Benzofurazan-(1)-oxide solution

Reagent preparation:
Dissolve 1.0 g of benzofurazan-(1)-oxide in 100.0 mL of ethanol. Reagent use:

Spray the plate with 2.0 mL of spraying solution B with the Derivatizer, yellow nozzle, spraying level 4, and record the images after 30 min.

3. Spraying solution E: NaOH solution

Reagent preparation:
Dissolve 4.0 g of NaOH in a mixture of 50.0 mL of de-ionized water and 50.0 mL of methanol.

Reagent use:
Spray the plate with spraying solution C with the Derivatizer (yellow nozzle, 3.0 mL, spraying level 4), heat at 150 °C for 30 s, and immediately record images. Spray the plate with spraying solution D with the Derivatizer (yellow nozzle, 3.0 mL, spraying level 3), and then with spraying solution E with the Derivatizer (yellow nozzle, 3.0 mL, spraying level 6), and record the images within the next 2 min.

Documentation

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

Densitometry

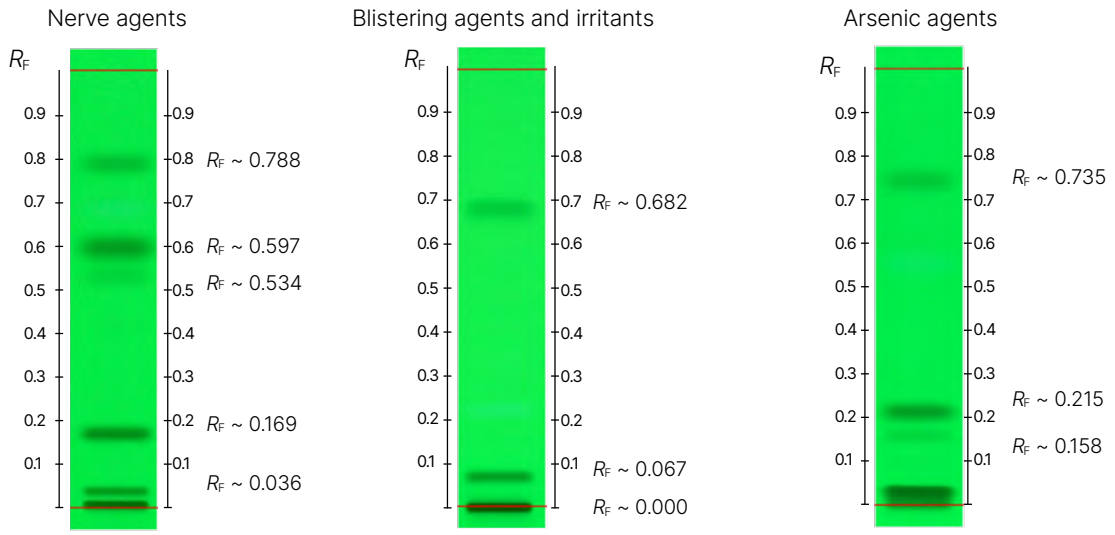
For the UHM, TLC Scanner 4 and *visionCATS*, absorbance measurement at 254 nm, slit dimension 5.00 mm x 0.20 mm, scanning speed 50 mm/s, and in fluorescence mode at 366>/400 nm. For the other substances, each standard is detected at their maximum of absorption as described in the following table.

Nerve agents	Fluorescence measurement at 540 nm (tungsten and deuterium lamps; no filter)
Blistering agents and irritants	Absorbance measurement at λMAX λMAX HD = 305 nm; λMAX TTA = 200 nm; λMAX CS = 305 nm; λMAX CN = 250 nm; λMAX CA = 240 nm; λMAX CB = 230 nm
Arsenic agents	Absorbance measurement at 230 nm (for L and DA) and 300 nm (for DM)

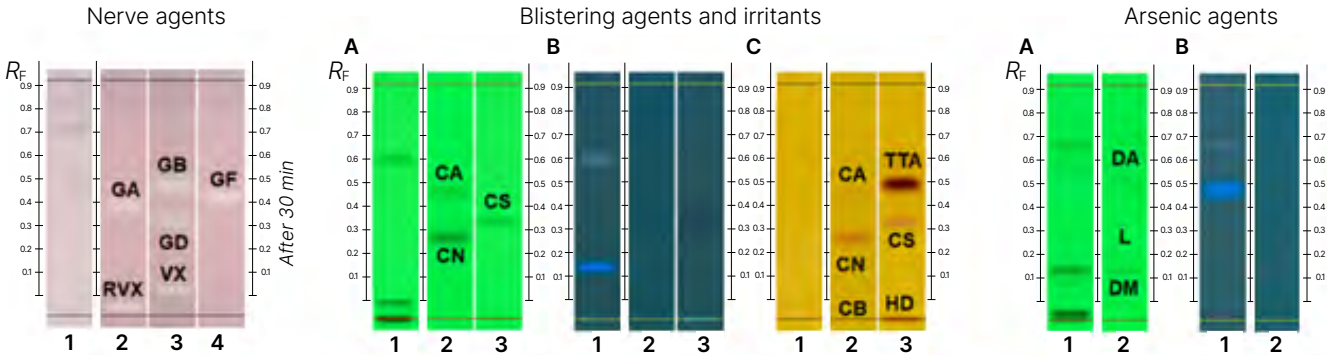
Results and discussion

For each method, the UHM was used as SST and the R_F values to obtain for each method are described as follows:

Forensics



R_F values to obtain for SST using the UHM for each method



Code	Name	R_F value
RXV	Russian VX	0.044
VX	-	0.108
GD	Soman	0.418
GA	Tabun	0.466
GF	Cyclosarin	0.510
GB	Sarin	0.555

Code	Name	R_F value
HD	Sulfur mustard	0.011
CB	Benzyl bromide	0.010
CN	2-Chloroacetophenpne	0.363
CS	-	0.438
CA	Bromobenzyl cyanide	0.561
TTA	HN-3	0.606

Code	Name	R_F value
DM	Adamsite	0.224
L	Lewisite	0.298
DA	Clark 1	0.639

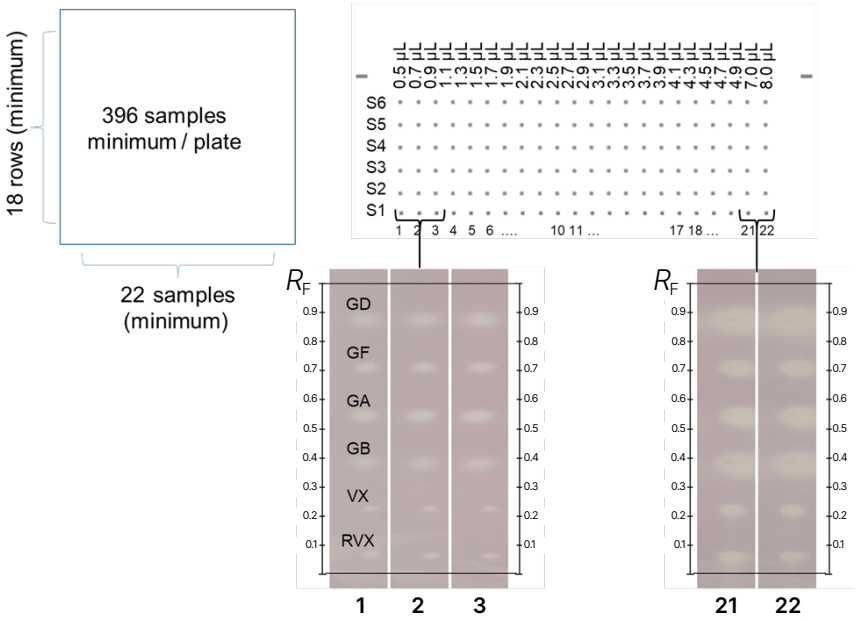
HPTLC chromatograms and R_F values for each nerve agent, blistering agents and irritants, and arsenic agents

For nerve agents, a large-scale untargeted screening of samples was developed, involving the detection of toxic substances without specific identification.

In this approach, each sample (utilizing reference substances in our example) was applied at different Y positions, forming a zone equivalent to a 1.0 mm band, with varying application volumes. In our example, the screening was applied on a 20 x 10 cm plate, but the screening could also be applied to a 20 x 20 cm plate.

Following the application, no development was conducted, but the entire plate underwent derivatization. Positive zones were observed as yellow against a pink/violet background. This test revealed that each nerve agent was still detectable at very low absolute quantities (amount on the plate):

- GA, VX, RVX < 0.25 ng
- GB < 0.125 ng
- GD < 0.025 ng
- GF < 0.01 ng



Protocol developed for large-scale untargeted screening of samples for detection of nerve agents (top), and example with reference substances in white light after derivatization (bottom). RVX (0.5 ng/µL) was applied at Y = 10 mm, VX (0.5 ng/µL) at Y = 20 mm, GB (0.25 ng/µL) at Y = 30 mm, GA (0.5 ng/µL) at Y = 40 mm, GF (0.02 ng/µL) at Y = 50 mm, and GD (0.05 ng/µL) at Y = 60 mm

Conclusion

The examples above show that HPTLC is a valuable tool for identifying nerve agents, blistering agents and irritants, as well as arsenic agents which are important for law enforcement and military personnel in preventing chemical warfare. HPTLC's format preserves the separated zones, allowing for further investigation including bioassays like acetylcholinesterase inhibition. Additionally, the use of HPTLC instruments reduces the need for analysts to physically interact with toxic samples, enhancing safety.

Further information is available on request from the authors.

Literature

[1] CAMAG Application note A-142.1: Identification and quantification of arsenics agents L, DA and DM by HPTLC.

[2] CAMAG Application note A-143.1: Identification and quantification of blistering agents and irritants HD, TTA, CS, CN, CA and CB by HPTLC.

[3] CAMAG Application note A-144.1: Identification and quantification of nerve agents RVX, VX, GD, GA, GF and GB by HPTLC, and methodology for a large-scale untargeted screening.

[4] T. K. T. Do *et al.*, J Chromatogr A (2021) 1638

Contact

Dr. Tiên Do, Sonnenmattstrasse 11, 4132 Muttenz, Switzerland, tien.do@camag.com

Comparative HPTLC fingerprinting of saffron samples for quality evaluation

Mr. Ramakant Yadav

Mr. Ramakant Yadav, an application specialist under the guidance of Akshay Charegaonkar (Managing Director), works at Anchrom Enterprises Pvt Ltd, Mumbai, India. The company specializes in instrumental Planar Chromatography and is renowned for its expertise in developing novel, quantitative, and regulatory-compliant analytical methods for a wide range of products, including pharmaceutical formulations, APIs, herbal products, food items, organic intermediates, and dyes. Mr. Yadav finds HPTLC advantageous due to its rapidity, ease of use, cost-effectiveness, and data outputs such as plate images both pre- and post-chromatographic derivatization, along with the ability to evaluate data through image, profile, and spectrum comparisons.

Introduction

Crocus sativus L. commonly known as saffron, is a perennial stemless herb that is widely cultivated in Iran, India and Greece. It is obtained by drying the stigma of *C. sativus* L., which belongs to the Iridaceae family. Saffron plays a pivotal role in modern and traditional medicine, it is utilized for the prevention and treatment of various diseases and has anti-hypertensive, antioxidant, antidepressant, and anti-inflammatory activity. This precious spice holds a broad spectrum of applications in the food and cosmetic industries, serving as both a flavoring and coloring agent. The quality of saffron is affected by various factors, such as cultivation regions, climate, drying process, and storage conditions. However, the high value of this product makes it very susceptible to economic adulteration, which involves the mixing of low-quality spices with saffron, the addition of plant materials, and the use of natural or artificial colorants to imitate the color of saffron.

HPTLC is widely implemented in the food industry as a convenient and low-cost approach for separations of different chemical components, such as adulterants and contaminants. It is well suited for adulteration studies, because it is inexpensive and time-saving. By HPTLC, 15-20 samples can be detected simultaneously on one plate in about 20-30 minutes. The solvent consumption is only about 20 mL for those 20 samples and little waste is produced. Hence a method was developed for comparison of marketed saffron samples with pure saffron sample (BRM) to find possible adulterants.

Standard solutions

100 mg of *Crocus sativus* L. (Saffron BRM) is dissolved in 10 mL of 70 % ethanol.

Sample preparation

100 mg of *Crocus sativus* L. (Saffron) marketed formulations are dissolved in 10 mL of 70 % ethanol. The samples are vortexed thoroughly and centrifuged at 3000 rpm for 5 min. After centrifugation, the supernatant was collected and used for the application.

Chromatogram layer

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

Sample application

2.0 and 5.0 µL of sample solutions and 2.0 and 5.0 µL of standard solutions are applied as bands with the Automatic TLC Sampler (ATS 4), 15 tracks, band length 8.0 mm, distance from the left edge 20.0 mm, track distance 11.4 mm, distance from the lower edge 8.0 mm.

Chromatography

Plates are developed in the ADC 2 with chamber saturation (with filter paper) for 20 min and after activation at 33 % relative humidity for 10 min using a saturated solution of magnesium chloride, development with ethyl acetate – methanol – water 18:4:3 (*V/V*) to the migration distance of 70 mm (from the lower edge), followed by drying for 5 min.

Herbal drug analysis

Post-chromatographic derivatization

The plate is pre-heated at 105 °C for 3 min using the TLC Plate Heater and is then sprayed with 3 mL of natural product A reagent (1 g of 2-aminoetheyl diphenylborinate in 200 mL of ethyl acetate) using the Derivatizer.

Documentation

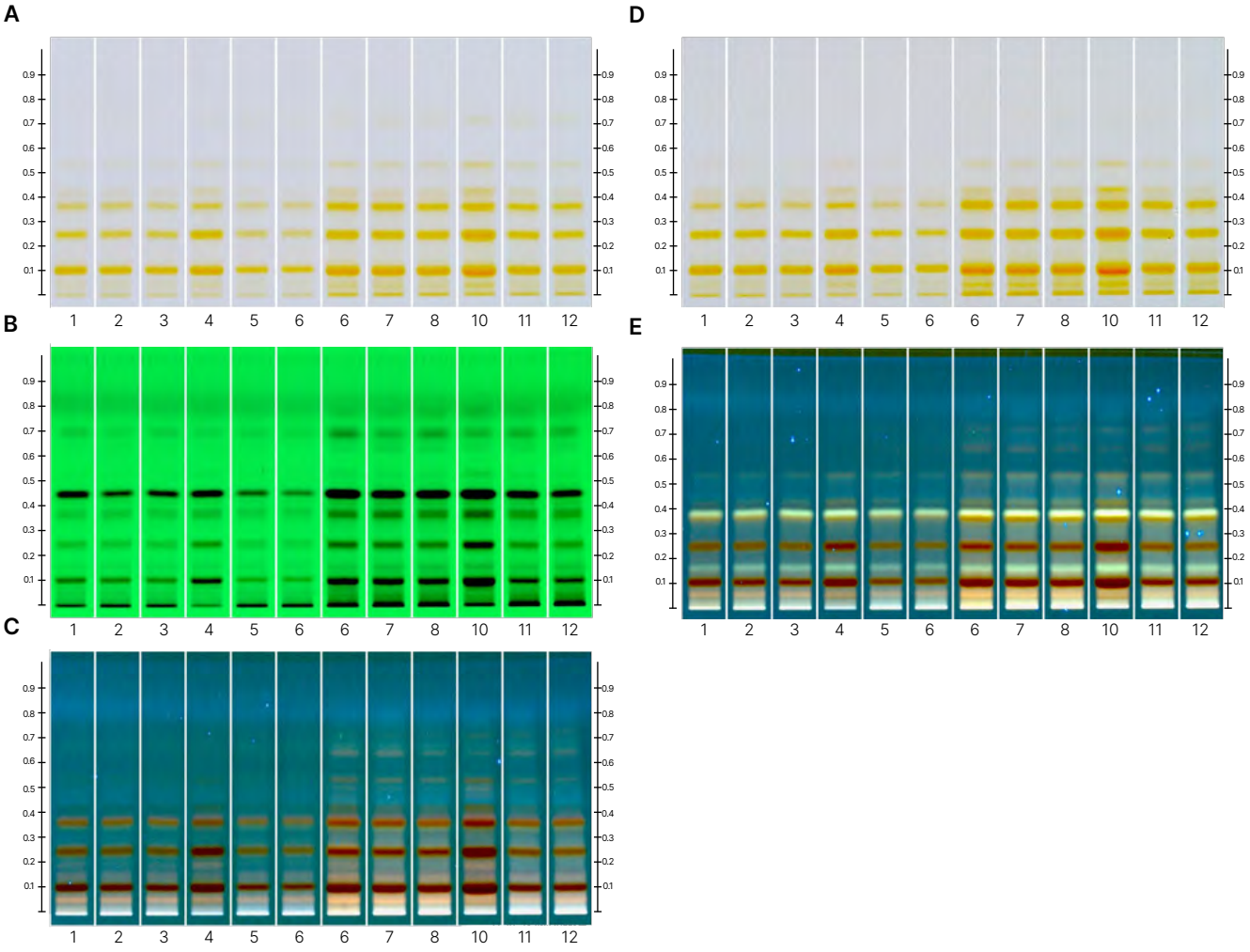
Images of the plate are captured with the TLC Visualizer in UV 254 nm, UV 366 nm, and white light.

Results and discussion

The analysis conducted on saffron samples involved a comparison between saffron (BRM) and saffron available in the market (branded and non-branded). Upon developing the chromatographic plate, it was observed that the fingerprints of all the saffron samples from the market were identical to that of the saffron (BRM). Notably, no adulterants were detected in any of the saffron samples, as there were no discernible colored bands observed apart from the characteristic fingerprint pattern.

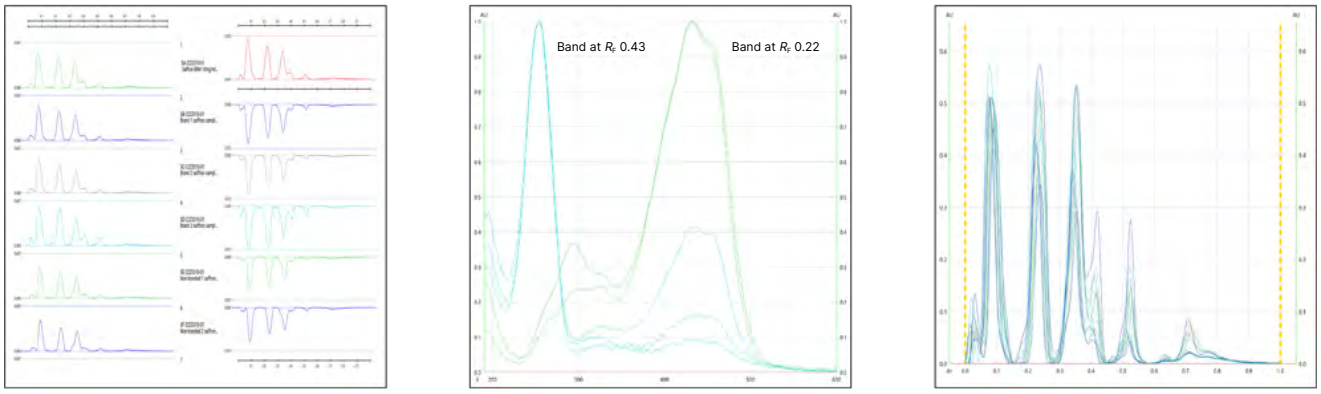
Further investigation involved taking spectra of the major bands detected in all the saffron samples for the purpose of comparison.

The results of this spectral analysis revealed that all the spectra from the different saffron samples, including both BRM and market-sourced saffron, matched identically. This suggests consistency and purity among the saffron samples, reinforcing their authenticity and quality.



HPTLC fingerprints: White light (A), UV 254 nm (B) & UV 366 nm (C) prior to derivatization, and white light after derivatization (D) and UV 366 nm after derivatization (E); Tracks 1 & 7: Saffron (BRM), tracks 2 & 8: marketed branded sample 1, tracks 3 & 9: marketed branded sample 2, tracks 4 & 10: marketed branded sample 3, tracks 5 & 11: marketed non-branded sample 1, and tracks 6 & 12: marketed non-branded sample 2

Herbal drug analysis



Left: Stack and flip view of saffron BRM and marketed saffron samples; middle: spectrum comparison of the two prominent zones found at R_F 0.22 and 0.43 in both the marketed saffron samples and the BRM; right: HPTLC peak profiles for saffron BRM and marketed saffron samples

Literature

[1] American Herbal Pharmacopoeia

Further information is available on request from the authors.

Contact

Mr. Akshay Charegaonkar, A-101, Shree Aniket Apartments, Navghar Road, Mulund East, Mumbai, Maharashtra 400081, India, hptlc@anchrom.in, www.anchrom.in

Application of Design of Experiments (DoE) on robustness studies and method evaluation of an HPTLC method for Arnica flower

Alina Kaya, Dr.-Ing. Margit Müller, Prof. Dr. Constanze Stiefel

The bachelor thesis described below was conducted at the Department of Analytical Research / Development at WALA Heilmittel GmbH in cooperation with Esslingen University of Applied Sciences, Germany.

Dr.-Ing. Margit Müller leads the team of Analytical Development Intermediate and Finished Products at WALA Heilmittel GmbH (Bad Boll, Germany). She and her team are responsible for analytical methods and specifications for medicinal products and plant-based active pharmaceutical ingredients including herbal drugs. HPTLC and TLC methods are widely used for identity testing in quality control, including stability testing of WALA products.

Prof. Dr. Constanze Stiefel teaches instrumental analysis at the Faculty of Science, Energy and Building Services at Esslingen University of Applied Sciences. Her research focuses on the application and development of chromatographic methods, including HPTLC and effect-directed analysis to determine bioactive compounds, contaminants and residuals in food and cosmetics.

Alina Kaya studied Chemical Engineering / Color and Coatings at Esslingen University of Applied Sciences. The shown study represents her bachelor thesis, concluding her Bachelor of Science degree.

Introduction

The presented study examines the implementation of software-based Design of Experiments (DoE) for robustness studies and method evaluation using the European Pharmacopoeia HPTLC method for identity testing of Arnica flower (monograph 1391, Ph. Eur. 11.0) as an example. DoE is expected to reduce practical effort in comparison to the traditional "one-factor-at-a-time" approach of experimentation. A statistically-based DoE approach aims at identifying significant factors and their interactions in relation to one or more response variables. Furthermore, it can be used to assess the robustness of the method against various factors or factor ranges.

The practical implementation of robustness studies is time-consuming. The use of DoE is intended to make these investigations more efficient by reducing practical effort while at the same time broadening method knowledge. Once a robust and valid parameter range has been identified by DoE, future method changes can be assessed with more confidence. The regulatory relevance of such variations can thus be reduced, which is advantageous because the testing of medicinal products must adhere either precisely to European Pharmacopoeia methods or require validation for every modified method. The use of DoE enables significant time and cost savings, making it highly interesting for pharmaceutical manufacturers.

Design of Experiments

The set-up of DoE-based experimental plans was carried out with the help of the software "Design Expert®" (version 22, StatEase, USA). Screening, characterization, and optimization studies were carried out.

A two-level factorial design was used for screening and characterization studies, investigating linear relations. Response surface methods were used for carrying out optimization studies, investigating more complex relationships such as quadratic relations. To take a closer look at the influence of the developing solvent components, a Mixture Design was used.

Standard solutions

Reference solution a: 1.0 mL of caffeic acid solution (1.0 mg/mL) and 1.25 mL of rutoside rihydrate solution (1.0 mg/mL) are diluted in methanol R to 5.0 mL.

Reference solution b: 500 µL of caffeic acid solution (1.0 mg/mL) and 625 µL of rutoside-trihydrate solution (1.0 mg/mL) are diluted in methanol R to 10.0 mL.

Reference solution c: 1.0 mL of chlorogenic acid solution (1.0 mg/mL) and 2.5 mL of hyperoside solution (1.0 mg/mL) are diluted in methanol R to 10.0 mL (used as system suitability test (SST)).

Sample preparation

2.00 g of powdered herbal drug (710) are extracted with 10.0 mL of methanol R, ultrasonically treated for 15 min, and filtered. The filtrate is used.

Chromatogram layer

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

Sample application

2.0 µL of sample and reference solutions are applied as bands with Automatic TLC Sampler (ATS 4), band length 8.0 mm, distance from left edge 20.0 mm, track distance 12.0 mm, distance from the lower edge 8.0 mm.

Chromatography

Plates are developed up to 70 mm (from the lower edge) in a saturated 10 × 10 cm twin trough chamber with formic acid R – water R – ethyl acetate R 6:9:90 (*V/V*), followed by drying for 5 min with a cold air dryer.

Post-chromatographic derivatization

Plates are derivatized using the Derivatizer. After heating the plate at 105 °C for 5 min, the plate is sprayed while still warm, with 2.0 mL of diphenylboryloxyethylamine in methanol R (10 g/L) and 2.0 mL of Macrogol 400 R in methanol R (50 g/L), blue nozzle, praying level 3. The plate is air dried for 5 min.

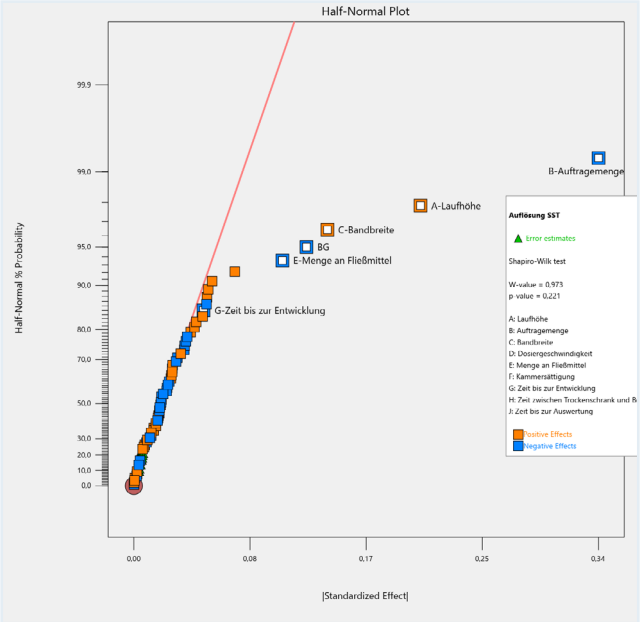
Documentation

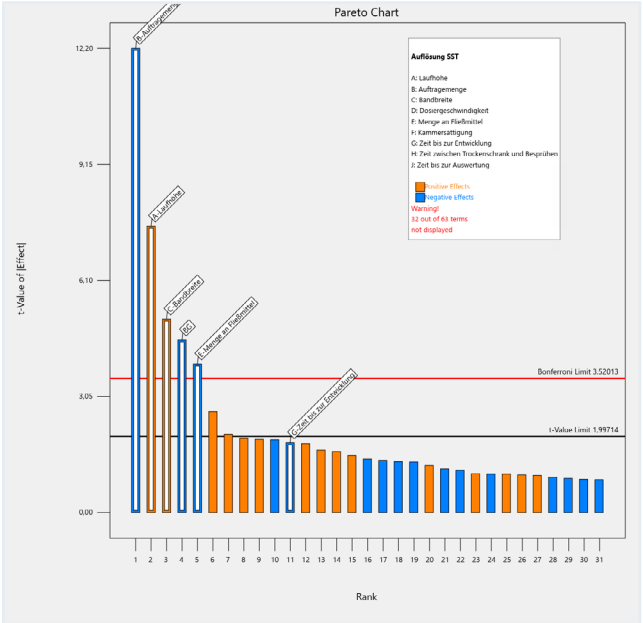
Images of the plate are captured with the TLC Visualizer in UV 366 nm after derivatization.

Results and discussion

In the first step of the investigation, critical risk factors were identified through a two-level factorial screening DOE design. As a result of the risk analysis, nine factors were identified as potentially critical for this HPTLC method. High level (+ 1) and low level (– 1) values of each risk factor were defined. The resolution between the two SST-components was selected as the response.

Before starting the experiments, a statistical power analysis was carried out using the software to ensure that the experimental plan had good predictive power. The power of a system should always be above 80 %, which was confirmed in this case. Experimental runs were performed in the laboratory, and response values were measured.The measured responses were entered into the Design Expert software against respective experimental runs and data analysis was performed using half normal plots and Pareto charts.

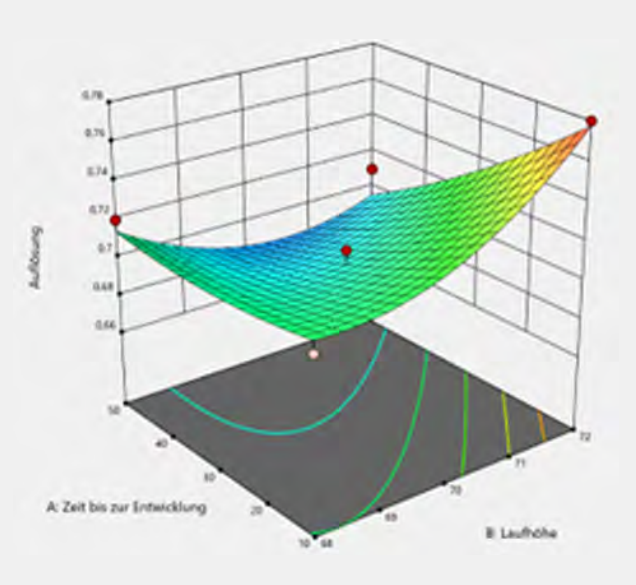




Half normal plot (left) and pareto chart (right) of two-level factorial screening design. Factors deviating from the red line of the half normal plot or the bars above the red line of the Pareto chart are statistically significant.

Herbal drug analysis

From the screening design, it was concluded that the application rate, development distance, volume of developing solvent, band length and the two-factor interaction between application rate and time until development are statistically significant. These identified risk factors need to be optimized to minimize their risk. Optimization was performed by DoE based on a central composite design. The central composite design was selected as response surface methodology to establish the relationship between the identified critical risk factors and resolution of the SST. Resolution values were added into the software against their respective experimental runs, and response surface analysis was performed with the help of variance analysis (ANOVA) and contour plots.

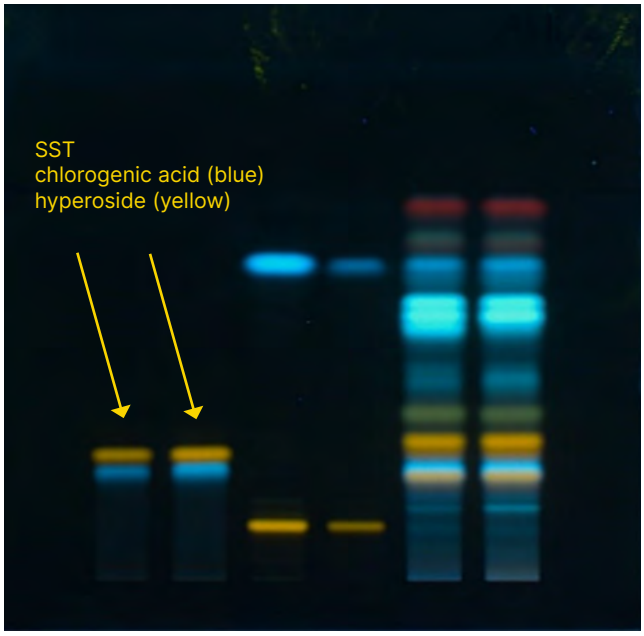


3D-contour plot of the experimental space

Although the optimization of significant factors was not successful in the context of this HPTLC-method, as no meaningful results could be generated, it can be said that the method is robust in the examined area because the resolution values of the different experiments changed only slightly.

For additional insights, an optimal mixture design study was carried out by investigating the robustness of the method with respect to formic acid and ethyl acetate concentration in the developing solvent. In addition to resolution of the SST components, the position of chlorogenic acid (substance of the SST) in the densitogram (expressed in mm) was selected as response parameter. With increasing amounts of ethyl acetate and decreasing amounts of formic acid, the resolution and the absolute position of chlorogenic acid decreased.

The application of DoE enables the efficient solution of different chromatographic problems in the field of TLC and HPTLC, in robustness studies of various method



SST resolution with the highest proportion of formic acid

SST resolution with the lowest proportion of formic acid

parameters, and in method validations, by creating systematic, precise experimental plans. The influence of various factors on any number of target variables can be investigated and evaluated in an experimental design. However, it must be noted that the number of tests increases with the number of factors to be investigated. The study showed that it was straightforward to assess the robustness of the selected TLC method across various factors. It was also possible to estimate the impact of method changes on the target variable within the area investigated by DoE.

Optimizing significant factors and minimizing their risks by determining a robust range was not possible for the presented method. However, this limitation may not apply to other methods. Various software programs offer a wide range of experimental design options. These include screening designs, response surface designs, mixture designs and many more. The generated data can be analyzed using a variety of statistical methods and analysis tools.

The results can be visualized through diagrams, graphs, and three-dimensional models. The results are statistically validated by the implementation of DoE-based experimental plans. However, it is essential to carefully consider the design of the experiment and the choice of factors and responses. The experimental conditions should be carefully controlled, and it should be ensured that the results are statistically valid and relevant to the intended field of application.

As a result, the thesis proved that by using a DoE-based approach the relevance of various robustness parameters and changes regarding developing solvent components can be assessed systematically and efficiently relating to the selected response parameter resolution of SST-components.

Literature

[1] Anderson, M. J. and Whitcomb, P. J. DOE simplified. Practical tools for effective experimentation. CRC Press. (2015)

[2] Anderson, M. J. RSM Simplified. Optimizing processes using response surface methods for design of experiments, second edition. CRC Press. (2017)

[3] Spangenberg, B. Quantitative Dünnschichtchromatographie. Eine Anleitung für Praktiker. Springer Spektrum (2014)

Further information is available on request from the authors.

Contact

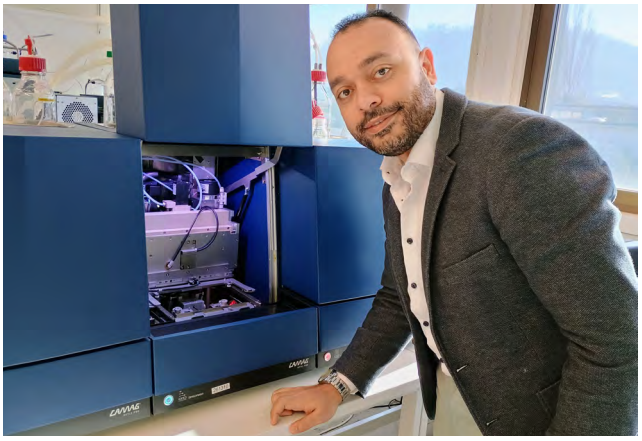
Dr.-Ing. Margit Müller, WALA Heilmittel GmbH, Badwasen 2(T1), 73087 Bad Boll, Germany, margit.mueller@wala.de

Prof. Dr. Constanze Stiefel, Hochschule Esslingen, Kanalstraße 33, 73728 Esslingen, Germany, constanze.stiefel@hs-esslingen.de

Alina Kaya, alinameryemkaya@gmail.com

Gas phase control in the HPTLC PRO Module DEVELOPMENT

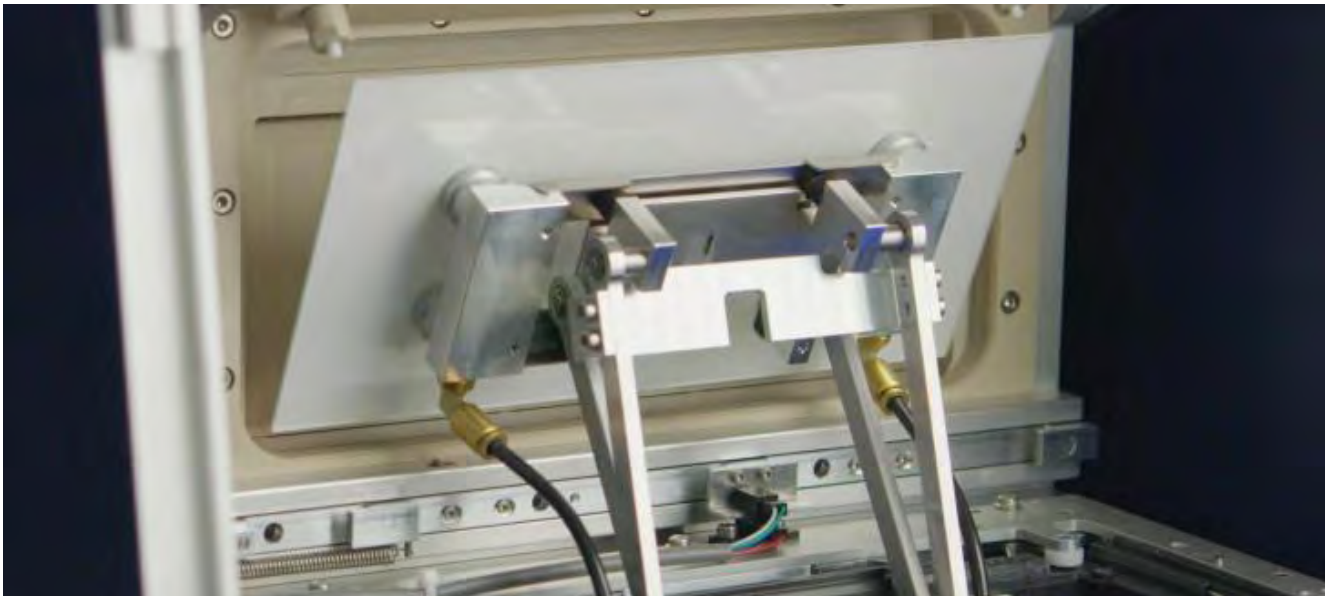
Dr. Ehab Mahran (CAMAG)



HPTLC is a straightforward analytical technique that offers numerous advantages. While the technique follows the same concept of separating mixture components between two phases (mobile phase and stationary phase), it differs from other liquid chromatographic techniques in the fact that a gas phase is present during and, indeed, influences the development process [1]. This property has always posed a challenging issue for controlling the outcome of the separation. Moreover, the fact that a broad spectrum of solvents can be used means gas phase control holds great promise for resolving complex matrix separations.

In order to investigate this, the Module DEVELOPMENT (a component of the CAMAG® HPTLC PRO System [2]) was employed in this study. The Module not only allows the generation and introduction of a gas phase of varying composition into the development chamber but also provides control over the timing and power settings of the pump used to build up the gas phase. The Module is equipped with three separate solvent bottles that enable the generation of gas phase from either the same solvents used for plate development or from different solvents. Additionally, the Module can be configured to introduce the generated gas phase at two distinct stages, prior to the start of the development (referred to as pre-conditioning) and/or during the development process (referred to as conditioning). These features provide useful tools to control the gas phase throughout the development process.

This study aims to investigate whether it is possible to manipulate the gas phase to attain a desired chromatographic separation. To achieve this objective, we sought to control the gas phase in a way that we can obtain R_F values based on the Universal HPTLC mix (UHM), a mixture of chemicals for system suitability testing, that are comparable to ($\Delta R_F \leq 0.05$) those previously measured using the ADC 2 [3].



The highly sophisticated development chamber featured in the fully automated HPTLC PRO Module DEVELOPMENT allows for the active control of the gas phase.

Standard solutions

The ready to use solution of UHM was prepared in house according to [4] and applied on track 8 (middle track) of each plate.

Chromatogram layer

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

Sample application

2.0 µL of UHM solution are applied as bands with the HPTLC PRO Module APPLICATION, 15 tracks, band length 8.0 mm, distance from the left edge 20.0 mm, track distance 11.4 mm, distance from the lower edge 8.0 mm. The default settings of methanol as sample solvent are used. The first rinsing step (bottle 1 solvent) is done with methanol – acetonitrile – iso-propanol – water – formic acid 250:250:250:250:1 (*V/V*) and the second rinsing step (bottle 2 solvent) is done with methanol – water 7:3 (*V/V*).

Chromatography

The plates are prepared in the HPTLC PRO Module DEVELOPMENT, using an activation step at 33 % relative humidity for 10 min with a saturated $MgCl_2$ salt solution. Throughout all experiments, the developing solvent used is ethyl acetate – toluene 1:9 (*V/V*) with a migration distance of 70 mm. The solvents used for generating a gas phase, the power pump values and durations of the gas phase for both pre-conditioning and conditioning are differently optimized according to each approach.

Documentation

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

Results and discussion

It is known that the gas phase surrounding the HPTLC plate during the development process can significantly influence the chromatographic separation. The HPTLC PRO Module DEVELOPMENT has a unique chamber design compared to the chambers used in the ADC 2 or for manual development. These differences can lead to changes in the rate of evaporation and the concentration of the developing solvent, which may result in differences in the pattern of separations. Therefore, it may be expected that the R_F values measured using the HPTLC PRO Module DEVELOPMENT will exhibit some deviations from those obtained through ADC 2 or manual development.

To explore the effects of the gas phase on compound separations, our goal in this study was to achieve R_F values similar to those obtained using the ADC 2 method.

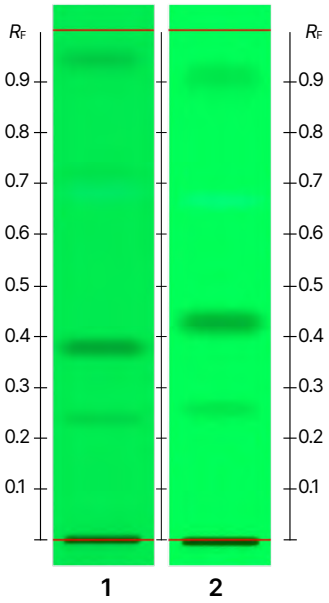
Key aspects of the study involve:

- Examining the impact of gas phase composition, while keeping the developing solvent and activation constant at 33 % rH.
- Setting a limit of ΔR_F at ± 0.05, meaning that the absolute difference between R_F values in the ADC 2 and the HPTLC PRO Module DEVELOPMENT should not exceed 0.05.

An initial experiment (HPTLC PRO M1) was conducted without using pre-conditioning or conditioning. In comparison to the ADC 2 results, the overall R_F values were different. However, compounds [e], [f], and [h] exhibited average R_F values within the specified control limits. Notably, ΔR_F was higher for compound [g] (~ 0.06).

These findings suggest that the migration pattern for all compounds does not behave uniformly. The development without the use of the gas phase leads to increased R_F values for compounds in the lower half of the plate, and to decreased R_F values for compounds in the upper half of the plate.

The challenge now is to control the retention of each of the four compounds individually on the plate solely based on gas phase control.



HPTLC chromatogram of UHM after development.
1: Experiment ADC 2, 2: Experiment HPTLC PRO M1

Based on this initial information, three methods were developed to evaluate the effect on the ΔR_F.

Methods used to study active gas phase control

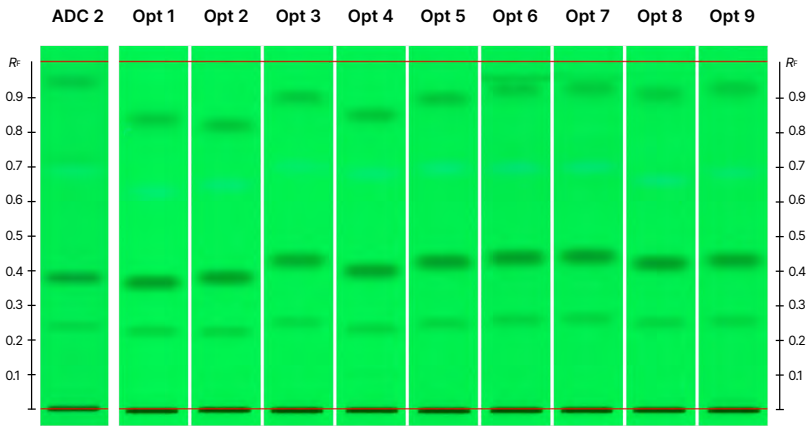
Method	Development	Pre-conditioning (solvent; pump power; duration)	Conditioning (solvent; pump power; duration)
ADC 2	activation at rH 33 % for 10 min using a saturated salt solution of $MgCl_2$ No saturation **DS**: ethyl acetate - toluene 1:9 (*V/V*)	N/A	N/A
HPTLC PRO M1	activation at rH 33 % for 10 min using a saturated salt solution of $MgCl_2$ **DS**: ethyl acetate - toluene 1:9 (*V/V*)	OFF	OFF
HPTLC PRO M2	activation at rH 33 % for 10 min using a saturated salt solution of $MgCl_2$ **DS**: ethyl acetate, toluene 1:9 (*V/V*)	ethyl acetate – toluene 1:9 (*V/V*); 50%; 10 s	ethyl acetate – toluene 1:9 (*V/V*); 50%; start after 600 s till 70 mm
HPTLC PRO M3	activation at rH 33 % for 10 min using a saturated salt solution of $MgCl_2$ **DS**: ethyl acetate - toluene 1:9 (*V/V*)	CPME – THF – H_2O – formic acid 40:24:1:1:1 (*V/V*); 35%; 5 s	ethyl acetate – toluene 1:9 (*V/V*); 30%; start after 50 mm for 45 s
HPTLC PRO M4	activation at rH 33 % for 10 min using a saturated salt solution of $MgCl_2$ **DS**: ethyl acetate - toluene 1:9 (*V/V*)	ethyl acetate – toluene 3:7 (*V/V*); 40%; 25 s	OFF

The method HPTLC PRO M2 focused on using the same solvent for both, the developing solvent and for gas phase generation. Initially, conditioning with the developing solvent was employed. However, the experiment revealed that initiating conditioning at various migration distances (while keeping the pump power constant) significantly affected the R_F values of individual substances.

For example, beginning conditioning at either 0 or 30 mm resulted in a substantial reduction of the R_F value for zones located in the upper part of the plate, while starting conditioning at 50 mm exhibited less impact on these zones. Consequently, we decided to initiate conditioning after 50 mm, leading to an improvement of the R_F values for most zones, except for compound [f], which required the use of a pre-conditioning step. Previous studies have shown that conditioning in normal phase HPTLC usually increases the R_F values and pre-conditioning lowers them.

Ultimately, increasing the pre-conditioning duration from 10 to 30 s corrected the R_F value for compound [f], but this came at the expense of reduced R_F values for compounds [g] and [h].

Those data highlight the various parameters that can be used to regulate the gas phase. It also reveals that substances respond differently to each given experimental condition, indicating that the chemical properties of the compounds play a role in regulating the gas phase.

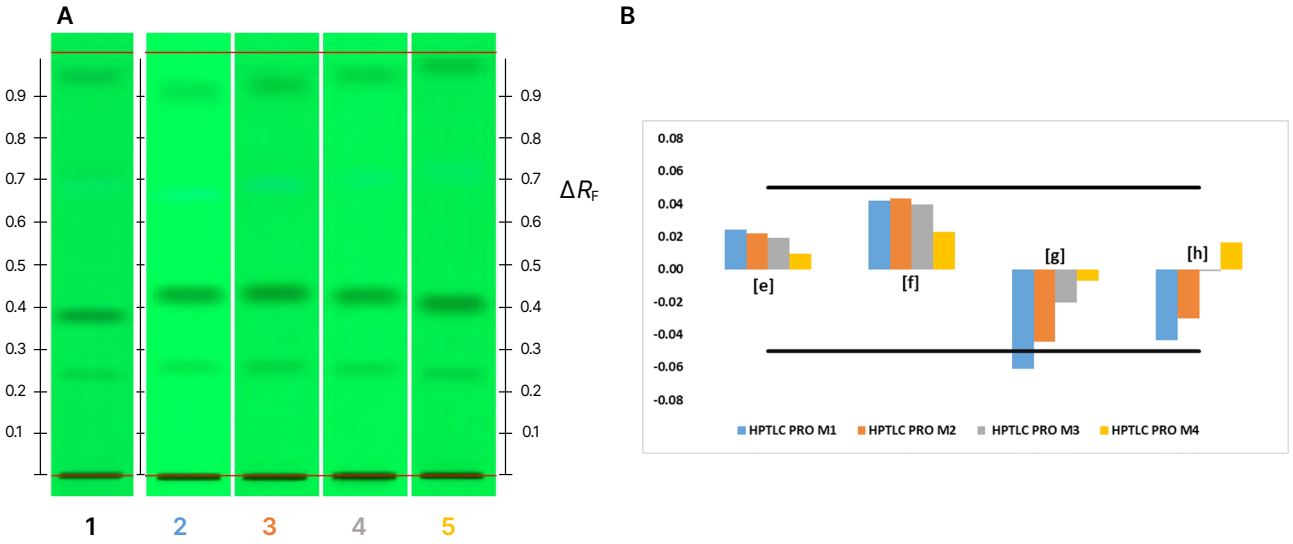


	Pre-conditioning (pump power; duration)	Conditioning (pump power; duration)
Opt 1	OFF	40%, 0 - 30 mm
Opt 2	OFF	40%, 30 - 50 mm
Opt 3	OFF	40%, 50 - 70 mm
Opt 4	OFF	40%, 30 - 70 mm
Opt 5	OFF	50%, 50 - 70 mm
Opt 6	OFF	40% after 600 s, 70 mm
Opt 7	30%, 10 s	40% after 600 s, 70 mm
Opt 8	30%, 10 s	40% after 600 s, 70 mm
Opt 9	50%, 10 s	50% after 600 s, 70 mm

HPTLC chromatograms show results obtained at different conditions with method HPTLC PRO M2

Similar optimization processes were employed in the other two approaches (optimization data not shown). However, in these two approaches we demonstrated how to control the gas phase with solvents that are different from the developing solvent. One approach involved entirely different solvents, adopted from [3] (referred to as HPTLC PRO M3), while the other maintained the same composition but different solvent proportions (referred to as HPTLC PRO M4).

Notably, the fourth approach (HPTLC PRO M4), which uses ethyl acetate – toluene 3:7 (*V/V*) for pre-conditioning, yielded the most favorable outcome. In this approach, no conditioning is required and in contrary to the common tendency for pre-conditioning to decrease R_F values (due to the known building of virtual fronts), our study revealed an anomalous outcome where R_F values for compounds other than [g] experienced an increase in R_F value. By exploring these alternative solvent combinations, we can expand our understanding about the effect of the gas phase composition and its subsequent impact on chromatographic performance.



*HPTLC chromatograms of the UHM after development with different conditions (**A**): track 1: ADC 2 (standard conditions), track 2: HPTLC PRO M1, track 3: HPTLC PRO M2, track 4: HPTLC PRO M3, track 5: HPTLC PRO M4; Control chart for ΔR_F (**B**)*

R_F values obtained from methods conducted in this study

Method	R_F (average n = 5)			
	[e]	[f]	[g]	[h]
ADC 2	0.232	0.384	0.725	0.951
HPTLC PRO M1	0.256	0.426	0.664	0.907
HPTLC PRO M2	0.254	0.427	0.680	0.921
HPTLC PRO M3	0.251	0.423	0.705	0.950
HPTLC PRO M4	0.242	0.407	0.718	0.967

Conclusion

This study emphasizes the essential role of the gas phase in regulating the development process and extends its significance beyond the establishment of standardized chromatographic procedures for HPTLC analysis.

Furthermore, this study shows, that it is possible to control the gas phase. By optimizing the composition of the gas phase, the pump power used to build up the gas phase, and the duration of the gas phase using the HPTLC PRO Module DEVELOPMENT, we demonstrated how the control of the gas phase allows the customization of the retention of each of the target compounds in specific regions of the chromatogram. This results in the achievement of the desired separation pattern through three distinct approaches.

This groundbreaking work highlights the critical role of the gas phase in controlling the development process, introducing new possibilities for strengthening and enhancing the selectivity of the gas phase on the development. These concepts, previously not fully explored, represent a significant step towards a deeper understanding of the complexities involved in pre-conditioning and conditioning processes within Thin-Layer Chromatography systems.

Literature

[1] E. Reich *et al.*, High-Performance Thin-Layer Chromatography for the Analysis of Medicinal Plants (2007).

[2] CAMAG CBS 123. Introducing CAMAG HPTLC PRO.

[3] T. K. T. Do *et al.* J. Planar Chromatogr. - Mod. TLC (2022) 299

[4] T. K. T. Do *et al.*, J Chromatogr A (2021) 1638

Further information is available on request from the authors.

Contact

Dr. Ehab Mahran, CAMAG, Sonnenmattstrasse 11,
4132 Muttenz, Switzerland, ehab.mahran@camag.com