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SEPTEMBER 2018

Preservatives in food

Other topics of this issue:

- **Effect-directed analysis of a water sample**
- **Identification of active ingredients in plant extracts**
- **Characterization of invertase activity**
- **Comprehensive HPTLC fingerprinting in quality control**

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cbs@camag.com
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Effect-directed analysis of a water sample

Lena Stütz and Dr. Stefan C. Weiss

For more than 10 years effect-directed analysis in combination with TLC has been used and advocated at the Laboratory for Operation Control and Research of Zweckverband Landeswasserversorgung in Langenau [1]. It began with the *Aliivibrio fischeri* inhibition assay for the detection of baseline toxicity. Now three more bioassays (end points: estrogenic, antibiotic and neurotoxic effects) have been applied. The method is used as a fast monitoring tool for investigation of raw and drinking water. Subsequently a method is described, which was developed in cooperation with Dr. Wolfgang Schulz and Dr. Rudi Winzenbacher (same institution) and Prof. Dr. Wolfgang Schwack, University of Hohenheim, Stuttgart.

Introduction

Through increasing influence of humans, the environment more and more gets polluted with anthropogenic trace substances. Many of these substances are not characterized and their effects on humans and the ecosystem are only inadequately clarified. Therefore it is very important to evaluate the eco(toxicological) relevance of this multitude of substances, classifying relevant from less relevant ones. Here, effect-directed analysis (EDA) can make a contribution as a combination of fractionation, bioassay and chemical analysis.

For fractionation of EDA, HPTLC has proven to be a particularly suitable method. In comparison to HPLC, HPTLC is an open separation system, thereby separated substances are solvent-free and the following *in-vitro*-bioassay can be performed directly. However, in comparison to HPLC, this method is limited due to the lower separation efficiency. To increase the separation efficiency of HPTLC, in this work a two-dimensional (2D) separation strategy with effect-directed detection was developed.

In the presented method, zones with detectable effect are eluted with the TLC-MS interface and the respective eluate (200 µL) is collected into a

sample vial. The acetylcholinesterase (AChE) inhibition assay for the detection of neurotoxic effects was performed in both separation dimensions. Through this selective procedure, fractions without AChE-inhibiting effects could be excluded after the first separation dimension (1D) and only the effective fractions were transferred for separation in the second dimension.

Standard solutions

For evaluation of the mobile phases, five standards mixed in methanol with a respective concentration of 10 ng/µL were applied. Additionally, a mix with 50 acetylcholinesterase (AChE)-inhibiting substances for spiking of a surface water sample with a concentration of 10 ng/µL of each substance was prepared.

Chromatogram layer

HPTLC LiChrospher silica gel 60 F_{254S} plates (Merck) were immersed twice in 2-propanol for 20 min. After drying at 120 °C the plates were predeveloped to the top edge with acetonitrile. Finally, the plates were heated again to 120 °C for 20 min.

Sample application

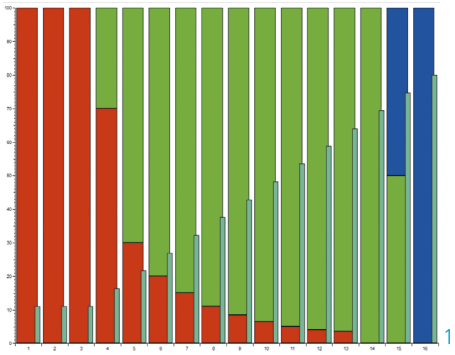
Bandwise application with Automatic TLC Sampler (ATS 4), band length 6 mm, distance from the lower edge 8 mm, distance from left edge 20 mm, application volume for standard solutions and spiked surface water sample 10 µL (first development) and 200 µL of collected fractions (second development)

Chromatography

First development is done in the AMD 2 with gradient (16 steps) up to a total migration distance of 80 mm. Isocratic development in the 2nd dimension is performed in the Automatic Development Chamber (ADC 2) with chamber saturation (15 min). The plate is activated for 1 min with a molecular sieve, preconditioned for 30 s, developed up to 70 mm and then dried (20 min with cold air).

AChE inhibition assay

The pH was adjusted to 7.5 by placing the HPTLC-plate in a NH_3 vapor saturated twin through chamber, followed by drying with vacuum for 10 min. Then the plate was immersed with the Chromatogram Immersion Device in the AChE solution (0.05 M Tris-HCl buffer, pH 7.8, with 0.1% bovine serum albumin), dipping speed 2 cm/s, dipping time 2 s. After subtraction of the abundant solution, incubation of the plate (5 min, 37 °C, >90% humidity) follows. After 6.5 min the substrate (0.5 g/L 3-indoxyl-3-acetate in aqueous solution with 4% DMSO) was applied (amount of reagent 580 µL, distribution 0.03 µL/mm^2) by spraying. Through active enzyme the substrate is cleaved to indoxyl, which reacts with oxygen to indigo white.



AMD gradient consisting of methanol – formic acid 100:0,05, dichloromethane and n-hexane [2]

Documentation

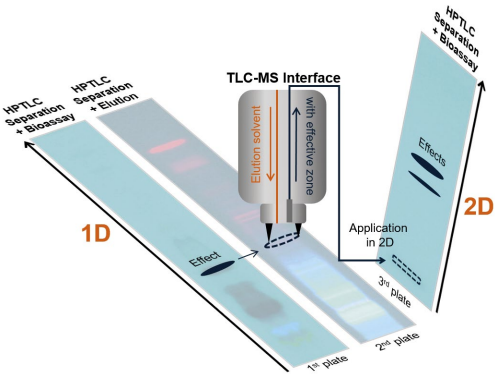
With the TLC Visualizer 10 pictures (1 picture/min, exposure time 800 ms) are taken under UV 366 nm. For the evaluation of the effect, the picture after 2 min was used.

Elution from the plate

With the TLC-MS Interface the zone is eluted with methanol for 1 min with a flow rate of 0.2 mL/min

Results and discussion

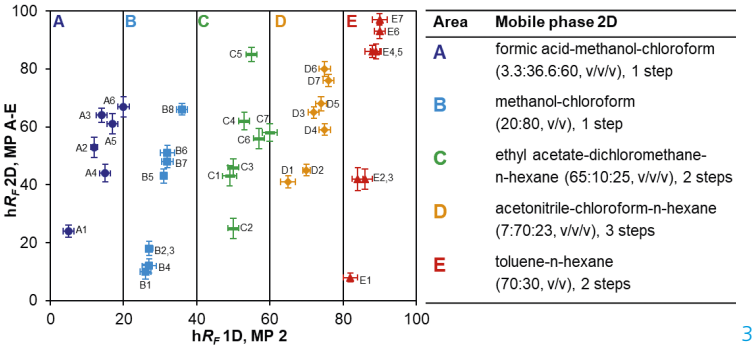
The first separation was performed in the AMD system with a 16-step gradient consisting of methanol (0.05% formic acid), dichloromethane and *n*-hexane – achieving a peak capacity of 30. With the development of a dynamic heart cut 2D separation approach the peak capacity was increased. Therefore, the first AMD chromatogram was parted in five equal hR_F-ranges. One effective substance was selected and the respective area investigated.



2

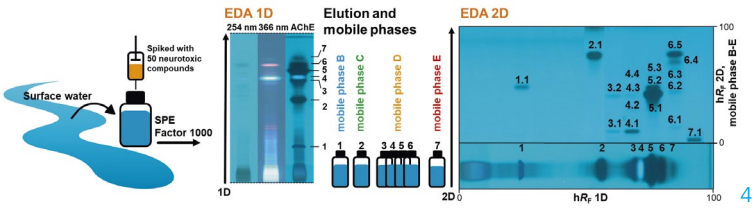
Image of the dynamic heart cut 2D approach with elution of the chromatogram zone from the parallel partial HPTLC plate that was only developed (no AChE assay) [2]

Based on 35 model substances the improvement of separation efficiency was shown. For example, it was possible to distribute seven substances, which occur in the first dimension in the hR_F-range of 80–100, through the second dimension to the hR_F-range of 7–91.



3

Spreading of the hR_F-range in the 2D, depicted based on 35 model substances [2]



4

Application of the approach for EDA of a spiked surface water sample

After method development the peak capacity of this dynamic approach was determined based on 125 substances. Therewith a peak capacity of 204 could be achieved, so that separation efficiency could be improved through the factor 7 in comparison to the 1D. The developed approach was applied to 50 AChE-inhibiting substances spiked with surface water sample. After AMD separation and the AChE inhibition assay in the 1D seven effective zones were selected. These were eluted and separated in the 2D with respect to the hR_F-range adapted mobile phase. With the AChE inhibition assay of the 2D, induced through increased peak capacity, obviously more effective zones could be detected compared to 1D (17 as opposed to 7). Moreover it was possible to assign 8 of the effective chromatogram zones clearly to one substance, based on their hR_F-values. After 1D the assignment succeeded only for 2 zones.

Through the developed dynamic 2D approach, the peak capacity of EDA could be increased by the factor 7, which enhanced assignment of substances in the spiked surface water. The required time of 2D-EDA of the spiked surface water sample amounted to 8 h. To identify also unknown effective substances in samples, a coupling of this method to further analytical techniques, like MS is possible. Through identification of effective substances, their relevance can be easier evaluated, which is also useful for determination of limit values.

[1] Weber, W. H., *et al.* CBS 94 (2005) 2

[2] Stütz, L., *et al.* Journal of Chromatography A 1524 (2017) 273

Further information is available from the authors on request.

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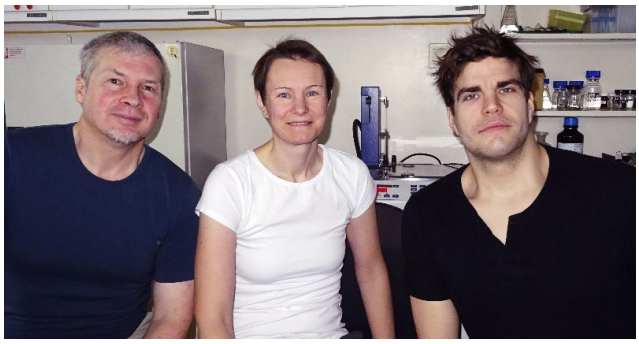
HPTLC-based effect-directed workflow in drug discovery



From left: Dr. Péter Ott, Dr. Ágnes Móricz and Dániel Krüzselyi

Dr. Ágnes Móricz, Dr. Péter Ott and Dániel Krüzselyi, Plant Protection Institute, Centre for Agricultural Research, Hungarian Academy of Sciences, aim to discover bioactive compounds from natural products using HPTLC-effect directed analysis (EDA) as a biomonitoring tool. They perform a bioassay-guided workflow that combines the analytical scale tracking and characterization with the preparative scale isolation and NMR identification of the compounds having the desired effect. This workflow interlacing with high-throughput effect-directed detection is successfully applicable for comprehensive plant extract screening. Part of the work was performed in cooperation with Prof. Dr. Gertrud Morlock, JLU Giessen, Germany.

Introduction

The search for suitable chemicals available to stop or reverse the effects of plant, animal and human diseases has initiated the quest for new drugs. The frequency of reports of antibiotic resistance is a global public health concern and the main driving force for the discovery of new antibiotics. There is a universal clamour for new effective substances applicable in the treatment of diabetes, *e.g.* Alzheimer's, hormonal and cardiovascular diseases. An infinite source of promising candidates of diverse chemical structure is to be found in nature, particularly the plant kingdom. Selecting the candidates may be challenging when individual active compounds are embedded in complex matrices. Effect-directed processes, including extraction, fractionation and purification steps, have been introduced that shorten the way to the component(s) with the desired activity. The core of these processes is a biomonitoring assay that interfaces the whole procedure and excludes the non-effective extracts and fractions from subsequent steps.

HPTLC-EDA fulfils the requirements for such a biomonitoring tool, enabling a fast, high-throughput and relatively cheap non-targeted screening for bioactive components from different extracts or fractions. The combination of HPTLC with MS allows a highly targeted characterization of the compounds of interest. In order to gain knowledge about the chromatographic, biological, biochemical and spectrometric behaviours, the workflow supports the isolation of the active compounds, which also allows their absolute identification by NMR.

Sample preparation

Dried and ground roots of fully flowered *Solidago virgaurea* L. (European goldenrod), and leaves of full flowering *Onopordum acanthium* L. (Scotch thistle) and *Helianthus annuus* L. (sunflower) were macerated for 24 h in ethanol or ethyl acetate (150 mg/mL). For HPTLC, each extract was 10-fold diluted with the respective solvent.

Chromatogram layer

HPTLC silica gel 60 F_{254} plates (Merck), 20 × 10 cm, for MS prewashed with methanol – water 4:1 and dried at 100 °C for 20 min

Sample application

Bandwise application with Automatic TLC Sampler (ATS 4) or Linomat 4, 7 or 8 mm bands, distance from lower edge 8 mm, application volume 1–10 µL/band

Chromatography

Extracts of European goldenrod, Scotch thistle and sunflower are separated in a Twin-Trough Chamber 20 × 10 cm with *n*-hexane – isopropyl acetate – acetic

acid 83:14:3, *n*-hexane – ethyl acetate 9:11 and *n*-hexane – isopropyl acetate – acetic acid 80:19:1, respectively, up to a migration distance of 70 mm, drying for 5 min. The chromatograms are neutralized with potassium hydroxide atmosphere for 4 h followed by a cold air stream for 20 min.

Documentation and postchromatographic derivatization

After development under UV 254 nm and UV 366 nm as well as under white light illumination after derivatization with vanillin-sulphuric acid reagent (200 mg vanillin, 1 mL sulphuric acid in 50 mL ethanol)

Effect-directed detection

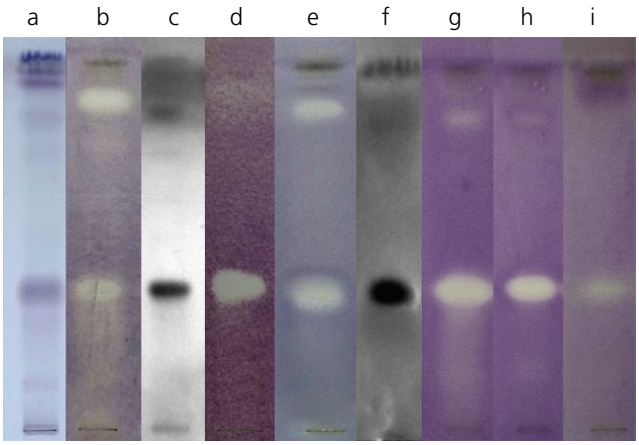
Bacterial or enzyme assays were used as described [3–5]: *Bacillus subtilis*, *Aliivibrio fischeri*, *Pseudomonas syringae pv. maculicola* and *Xanthomonas euvesicatoria*, *Lactobacillus plantarum* (ATCC 8014), *Staphylococcus aureus* (ATCC 29213), methicillin resistant *S. aureus* (MRSA4262) and *Escherichia coli* (ATCC 25922) as well as acetyl- and butyrylcholinesterase.

HPTLC-MS

Mass spectra are recorded with a modified HPTLC-DART-MS system in negative and positive ionization mode in the range of *m/z* 70–1000. For comparison, zones are eluted with a TLC-MS Interface using an oval elution head (4 × 2 mm) into a hybrid quadrupole Orbitrap mass spectrometer and the HPTLC-ESI-HRMS spectra are recorded in negative and positive ionization mode in the range of *m/z* 50–750 [3–5].

Results and discussion

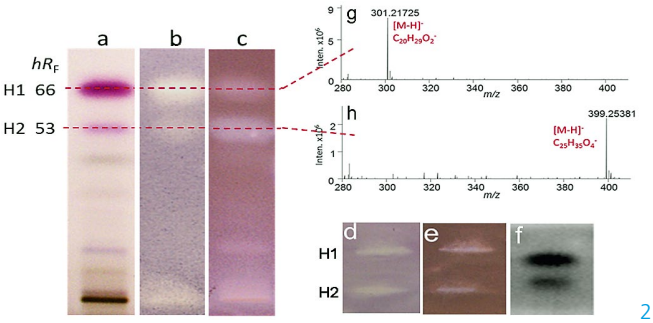
The initial step of the process was a HPTLC separation of the compounds in the crude extracts. Then the separated components were subjected to EDA. The subsequent characterization of the active zones was carried out by chemical derivatization, densitometry and MS. From the samples analyzed [1–5], three plant extracts were presented for illustration of the non-targeted screening for bioactive compounds. Exemplarily, the Scotch thistle leaf zone at hR_F 37 showed activity against all tested bacterial strains [3].



1

HPTLC chromatograms of antibacterial compounds in Scotch thistle leaf extract, documented under white light after derivatization with vanillin sulphuric acid reagent (a) as well as bioautograms using B. subtilis (b), A. fischeri (c), L. plantarum (d), X. euvesicatoria (e), P. maculicola (f), S. aureus (g), MRSA (h), and E. coli (i) (reprinted from [3] with permission)

As a second example, components of sunflower leaf extract displayed antibacterial activity and acetyl-/butyrylcholinesterase inhibition at hR_F 53 (H2) and 66 (H1) [4]. Exemplarily, the recorded HPTLC-ESI-HRMS spectra of the zones H1 and H2 in the sunflower leaf extract are depicted.

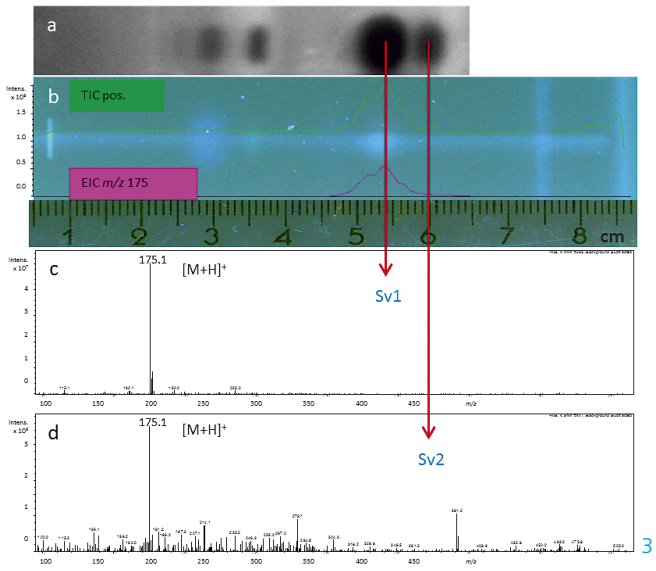


2

HPTLC chromatogram of active compounds in sunflower leaf extract, documented under white light after derivatization with vanillin–sulphuric acid reagent (a) as well as bioautograms using B. subtilis (b) and acetyl- (c; only applied, d), butyrylcholinesterase (e, applied only) and A. fischeri (f); HPTLC-ESI$^-$-HRMS full scan spectra of zones H1 (g) and H2 (h) (reprinted from [4] with permission)

As a last example, two characteristic antibacterial zones in European goldenrod root were revealed at hR_F 66 (Sv1) and 77 (Sv2). Sv1 also showed acetylcholinesterase inhibition [5]. The whole track of goldenrod was scanned by DART-MS and the recorded HPTLC-DART-MS spectra are shown. If compared to HPTLC-ESI-HRMS spectra, only the zones Sv1 and Sv2 were observable with DART-MS.

With both MS techniques, the same protonated molecule was obtained for both zones, confirming both compounds to be isomers.



3

HPTLC bioautogram of antibacterial compounds in European goldenrod root using A. fischeri (a) and HPTLC-DART-MS scan along the track (b, documented at UV 366 nm) with mass spectra of zones Sv1 (c) and Sv2 (d) (reprinted from [5] with permission)

After preparative isolation of selected active zones in the three samples and NMR identification, five active compounds were assigned: onopordopicrin from Scotch thistle leaf [3], (-)-kaurenoic acid and angeloyloxygrandifloric acid from sunflower leaf [4], and 2*Z*,8*Z*- and 2*E*,8*Z*-matricaria ester from European goldenrod root [5]. By this, HPTLC has been proven as a comprehensive tool for screening of plant materials for new active compounds.

[1] Móricz, Á.M. *et al*. J. Chromatogr. A 1543 (2018) 73–80
[2] Móricz, Á.M. *et al*. J. Chromatogr. A 1422 (2015) 310–317
[3] Móricz, Á.M. *et al*. J. Chromatogr. A 1524 (2017) 266–272
[4] Móricz, Á.M. *et al*. J. Chromatogr. A 1533 (2018) 213–220
[5] Móricz, Á.M. *et al*. Anal. Chem. 88 (2016) 8202–8209

Further information is available on request from the authors.

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4

CAMAG Automatic TLC Sampler (ATS 4)

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Customer training provided by CAMAG Berlin

Thorsten Schleuter (Head Sales), Margit Werther (former Head CAMAG Laboratory Berlin), Dr. Konstantinos Natsias (Director), Dr. Klaus Zieloff (Course instructor)

Martin Beetz (Head CAMAG Laboratory Berlin since the retirement of Ms. Werther in 2018)

As the daughter company of the Swiss headquarters, CAMAG Berlin is committed to promoting planar chromatography in Germany, Austria, Belgium and The Netherlands.

Since customer training and knowledge transfer are the life blood of the growth of planar chromatograpy, the competent Berlin team lead by Dr. Konstantinos Natsias offers a variety of activities aimed at this goal.

The most effective training lies with the numerous in-house courses at the customer site held by the CAMAG Berlin team for more than 15 years, Predominantly, these courses are tailored for the specific requirements of the individual customer.

Another very effective type of sales support are feasibility studies for both existing and potential customers, in order to determine and demonstrate whether planar chromatography offers advantages for a certain analytical task. These studies are offered by the CAMAG Berlin laboratory and can be performed in the presence of the customer, this way providing even more training.

While general training courses for planar chromatography held at the Swiss headquarters in Muttenz are usually in English, CAMAG Berlin offers such course in German. The next course will be held 16–17 October 2018 and will deal with error prevention in HPTLC, a subject that is timeless and one that is crucial in perfecting techniques and eliciting critical thinking. For details, see **www.camag.com**

Do you have ideas, how we can help you optimize planar chromatography in your lab? We are looking forward to receiving your suggestions.

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Remarks about abstracts newly added to the CCBS database with this CBS issue

In this issue, the CCBS collection of abstracts has been extended by 91 abstracts. Among the broad range of applications, the analysis of secondary metabolites has been topical. Several aspects to be addressed came up during the editing of the abstracts. The usage of proper terms is the basis of our scientific communication, as mentioned in CBS 117. Per definition, the term *bioautography* is used for biological assays in combination with TLC/HPTLC. Chemical assays, as given for the 2,2-diphenyl-1-picrylhydrazyl assay, are effect-directed assays. For quantitative measurements, the wavelength of 254 nm is only equal to the absorbance maximum of the analytes in very rare cases (*e.g.*, for parabens). Thus, it is recommended to record the UV/Vis spectrum of a compound first, in order to find the optimal measurement wavelength (absorbance maximum). Then, the calibration range should be as wide as possible, starting from the LOQ. A narrow calibration range of 1:2 (600–1800 ng/zone) does not make sense, particularly as the LOQ was reported to be 44 ng/zone. Instead, it is recommended to enlarge the calibration range to be 1:30 (40–1600 ng/zone), if the signal detection is not saturated at the highest concentration level. And last, but not least, calibration functions can be polynomial, as the signal obtained by diffuse reflection in TLC/HPTLC does not adhere to the Beer–Lambert–Bouguer law. If such aspects are considered, the HPTLC results are reported to be comparable to those obtained by a validated HPLC method. Method comparisons are recommended to verify the results!

Dear friends

The online CCBS database with its fast and effective search for keywords as well as the electronic versions of the CBS issues have become a helpful tool. Both are frequently used for searching the latest information on new TLC/HPTLC methods. The internet access for this free-of-charge scientific support has summed up to about 10 000 accesses at the CAMAG homepage a year.

Selected applications are headed as *Planar Chromatography in Practice* and give insight in the worldwide use of the TLC/HPTLC technique. Among others in this CBS issue, effect-directed analysis in drug discovery or water analysis, or characterization of the invertase activity are good examples of how the unique potentials of HPTLC can be exploited.

Since CBS 113, the four pages at the center part of each printed are reserved for information from the scientific community. On the fourth page in this issue we report on the 20th jubilee of the French *Club de Chromatographie sur Couche Mince*.

Find latest information on the next HPTLC symposium in Bangkok, 28–30 November 2018 and visit www.hptlc.com.

Kind regards

Gertrud Morlock
cbs@camag.com

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THE CBS CLASSIFICATION SYSTEM

1. **Reviews and books**
   a) Books on TLC
   b) Books containing one or several chapters on TLC
   c) Books containing frequent TLC information spread over several chapters of other information
2. **Fundamentals, theory and general**
   a) General
   b) Thermodynamics and theoretical relationship
   c) Relationship between structure and chrom. behaviour
   d) Measurement of physico-chemical and related values
   e) Optimization of solvent systems
   f) Validation of methods
3. **General techniques** (unless they are restricted to the application within one or two classification sections)
   a) New apparatus/techniques for sample preparation
   b) Separation material
   c) New apparatus for sample application/dosage
   d) New apparatus/techniques for chromatogram development
   e) New apparatus/techniques for pre- or post-chromatographic derivatization
   f) New apparatus/techniques for quantitative evaluation
   g) New apparatus/techniques for other TLC steps (distinguished from section 4)
4. **Special techniques**
   a) Automation of sample preparation/application
   b) Automation of complex chromatogram developing techniques
   c) Automation, computer application in quantitative chromatogram evaluation
   d) Combination of TLC with other chromatographic techniques
   e) Combination of TLC with other (non-chromatographic) techniques...MS, IR...etc.
5. **Hydrocarbons and halogen derivatives**
   a) Aliphatic hydrocarbons
   b) Cyclic hydrocarbons
   c) Halogen derivatives
   d) Complex hydrocarbon mixtures
6. **Alcohols**
7. **Phenols**
8. **Substances containing heterocyclic oxygen**
   a) Flavonoids
   b) Other compounds with heterocyclic oxygen
9. **Oxo compounds, ethers and epoxides**
10. **Carbohydrates**
    a) Mono- and oligosaccharides, structural studies
    b) Polysaccharides, mucopolysaccharides, lipopolysaccharides
11. **Organic acids and lipids**
    a) Organic acids and simple esters
    b) Prostaglandins
    c) Lipids and their constituents
    d) Lipoproteins and their constituents
    e) Glycosphingolipids (gangliosides, sulfatides, neutral glycosphingolipids)
12. **Organic peroxides**
13. **Steroids**
    a) Pregnane and androstane derivatives
    b) Estrogens
    c) Sterols
    d) Bile acids and alcohols
    e) Ecdysones and other insect steroid hormones
14. **Steroid glycosides, saponins and other terpenoid glycosides**
15. **Terpenes and other volatile plant ingredients**
    a) Terpenes
    b) Essential oils
16. **Nitro and nitroso compounds**
17. **Amines, amides and related nitrogen compounds**
    a) Amines and polyamines
    b) Catecholamines and their metabolites
    c) Amino derivatives and amides (excluding peptides)
18. **Amino acids and peptides, chemical structure of proteins**
    a) Amino acids and their derivatives
    b) Peptides and peptidic proteinous hormones
19. **Proteins**
20. **Enzymes**
21. **Purines, pyrimidines, nucleic acids and their constituents**
    a) Purines, pyrimidines, nucleosides, nucleotides
    b) Nucleic acids, RNA, DNA
22. **Alkaloids**
23. **Other substances containing heterocyclic nitrogen**
    a) Porphyrins and other pyrroles
    b) Bile pigments
    c) Indole derivatives
    d) Pyridine derivatives
    e) other N-heterocyclic compounds
24. **Organic sulfur compounds**
25. **Organic phosphorus compounds** (other than phospholipids)
26. **Organometallic and related compounds**
    a) Organometallic compounds
    b) Boranes, silanes and related non-metallic compounds
    c) Coordination compounds
27. **Vitamins and various growth regulators** (non-peptidic)
28. **Antibiotics, Mycotoxins**
    a) Antibiotics
    b) Aflatoxins and other mycotoxins
29. **Pesticides and other agrochemicals**
    a) Chlorinated insecticides
    b) Phosphorus insecticides
    c) Carbamates
    d) Herbicides
    e) Fungicides
    f) Other types of pesticides and various agrochemicals
30. **Synthetic and natural dyes**
    a) Synthetic dyes
    b) Chloroplasts and other natural pigments
31. **Plastics and their intermediates**
32. **Pharmaceutical and biomedical applications**
    a) Synthetic drugs
    b) Pharmacokinetic studies
    c) Drug monitoring
    d) Toxicological applications
    e) Plant extracts, herbal and traditional medicines
    f) Clinico-chemical applications and profiling body fluids
33. **Inorganic substances**
    a) Cations
    b) Anions
34. **Radioactive and other isotopic compounds**
35. **Other technical products and complex mixtures**
    a) Surfactants
    b) Antioxidants and preservatives
    c) Various specific technical products
    d) Complex mixtures and non-identified compounds
36. **Thin-layer electrophoresis**
37. **Environmental analysis**
    a) General papers
    b) Air pollution
    c) Water pollution
    d) Soil pollution
38. **Chiral separations**

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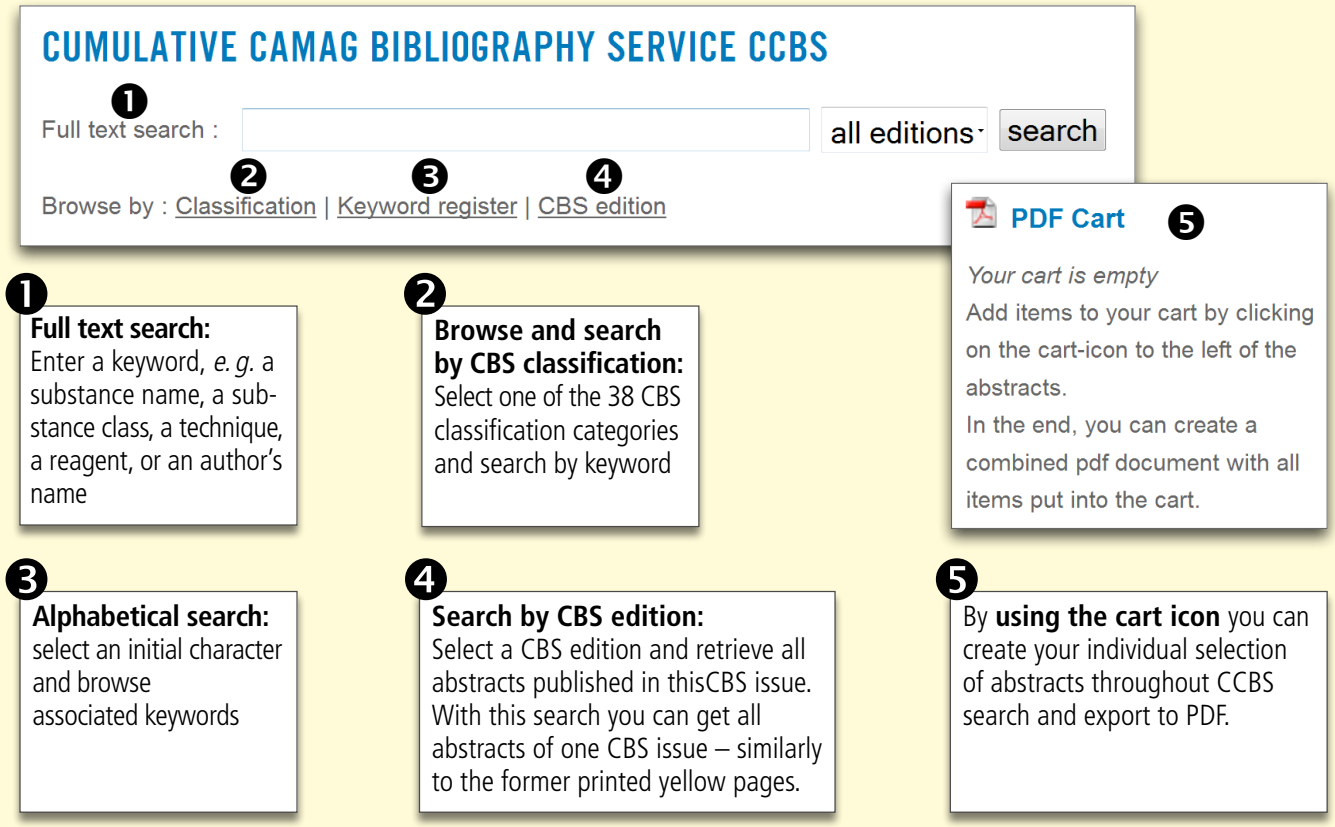


With the Cumulative CAMAG Bibliography Service (CCBS) Online Search, you can directly search for information within the CAMAG website. The CCBS covers more than 11'000 abstracts of TLC/HPTLC publications between 1982 and today. Providing reports was established by Dr. Dieter Jänchen in 1965 and the workflow and form evolved over the years. Manually written and transferred into the printed CBS form, it was set-up as a database in 1997. With CBS 93 in 2004, the electronic database for download was introduced and with the CBS 113 in 2014, the online search.

The database covers most relevant scientific journals in the field of Planar Chromatography including also various non-English publications in German, French, Spanish, Portuguese and Chinese. The CCBS features additional practical information for the analyst in the lab, for example details on the mobile phase or the detection. With CCBS the analyst is able to find relevant TLC/HPTLC publications which might be helpful for solving a particular analytical question.

Visit **www.camag.com/ccbs** and choose one of the following search options: full text search or search by CBS classification system or by alphabetical register or by CBS edition. For classical full text search, just enter a keyword in the search box, *e. g.*, a substance name, a substance class, an analytical technique, a reagent, or an author's name, and find all related publications throughout the CCBS.

Alternatively, you can choose to browse by one of the 38 CBS classification categories and search by keyword. The alphabetical search allows selecting an initial character and browsing associated keywords. When browsing by CBS edition, you can retrieve all abstracts published in the corresponding CBS issue.

To create your individual selection of TLC/HPTLC abstracts add your preferred publications to the PDF cart and download these selected articles in one single PDF file.

20th jubilee of the French *Club de Chromatographie sur Couche Mince* (CCCM)

In 1998 the French TLC Club was founded by representatives from industry and academia to bring scientists together for a fruitful exchange of knowledge in HPTLC. Since the number of scientists employing HPTLC was low as compared to HPLC, scientists appreciated these meetings for sharing knowledge instead of competing with each other. In every meeting, some scientists presented their work in order to discuss the results with each other. The Club was/is a good platform for expert lecturers to teach HPTLC as a modern analytical technique.

The first meeting took place near Lyon at Hoechst-Marion-Roussel in Neuville-sur-Saône on 10 June 1999. At that time the elected board consisted of Louis Vicard (Sanofi) as treasurer, Professor Dr. Philippe Berny (Veterinary School of Lyon) as secretary and Pierre Bernard-Savary (at this time VWR) as chairman. Since then the CCCM has met twice a year at various academic or industrial sites in France. Each meeting consists of a first day with a specific scientific topic, followed by a nice get-together, and a second day with presentations by invited guest speakers as well as users working in different application fields. Most of the presentations are still available online at **www.clubdeccm.com**. One of the latest presentations is published in this CBS on pages 9–11 by David Da Silva, ICOA Orléans.

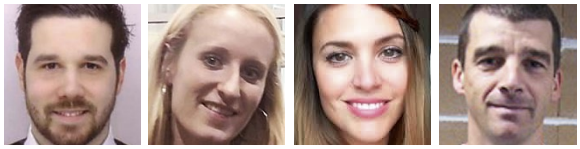
By the Committee at the International Symposium for HPTLC, the CCCM was selected as future Organizer, and in 2003 organized the next International Symposium in Lyon. Around 200 to 300 participants have attended the past symposia which take place every three years in Europe. Presently, the International Symposium will take place every year at alternating locations in Europe, Asia and America – in November 2018 in Bangkok, Thailand, in 2019 in Boulder Colorado, USA, and in 2020 in Ljubljana, Slovenia. Further details are available at **www.hptlc.com**.

The continued appreciation of the CCCM is due to the untiring will and commitment of all its members, between 50 and 100 scientists. One particular trend is the consistently high number of motivated students, which give the feeling that the members are getting younger … or is it just that we are getting older?

Pierre Bernard-Savary
CCCM President and Co-founder

Contact: **clubccm@hptlc.com**

Characterization of the invertase activity and identification of botanical substrates by densitometry and MALDI-TOF MS



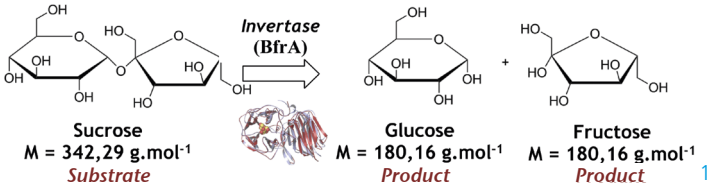
From left: Dr. David Da Silva, Dr. Justine Ferey, Sophie Bravo-Veyrat, Prof. Dr. Benoit Maunit

The different topics studied at the Institute of Organic and Analytical Chemistry (ICOA) are directed towards the design, synthesis and analysis of small molecules likely to have biological activities. The research group Analytical Strategy, Affinities and Bioactives is well known for the development of innovative methodologies aimed to extract, isolate and identify natural compounds from various plants. Depending on the nature of targeted molecules, phytochemical analysis of extracts is carried out by developing optimized, complementary and efficient chromatographic methods like HPLC, GC, SFC and HPTLC.

Introduction

Invertase is a widely spread enzyme of microorganisms and plants, essential for cell differentiation and sucrose regulation. Although invertases have been characterized in enzymology, it is necessary to improve analytical methods for finding their kinetic parameters (maximum velocity V_{max} and Michaelis constant K_M) through determination and quantification of their sugar products.

TLC analysis was employed for quantification of glucose and fructose as products of the invertase (β-D-fructofuranosidase from *Leishmania major*, BfrA) reaction with sucrose and for identification of botanical substrates (saccharides that can be degraded by invertase). TLC has been the method of choice for kinetic studies of invertases, due to the low sample/solvent volumes required, easy and fast operation, low costs, reliable and robust workflow, repeatable results and parallel analysis of samples. MALDI-TOF-MS was used to characterize the saccharides.



Sucrose hydrolysis by invertase [2]

Standard solutions and enzyme reaction

60 µL of invertase (0.02 g/L BfrA in ammonium formate buffer, pH = 6.4, 50 mM) and 60 µL of each sucrose solution (10 to 1000 mM in the same buffer) is mixed and incubated at 22 °C for 20 min. The reaction is stopped by heating at 95 °C for 5 min. Each solution is diluted with acetonitrile and water 1:1, containing α-L-rhamnose as internal standard (20 mM).

Sample preparation

1 g of a powdered plant is microwave-extracted with 7 mL ethanol 3 times for 30 s (irradiation power 1000 W) and centrifuged for 10 min. The supernatant is filtered (0.45 µm). 40 µL of plant extract are mixed with 40 µL of invertase solution (0.04 g/L in ammonium formate buffer). As control, each plant extract is 1:1 mixed with ammonium formate buffer without invertase. The reaction is stopped after sample application. The plate is kept at room temperature for 2 h.

Chromatogram layer

TLC aluminium foils silica gel 60 F_{254} (Merck), 20 x 20 cm and cut to 20 x 10 or 10 x 10 cm for development, prewashed with methanol and impregnated by immersion in a mixture of 0.1 M sodium bisulphite and 0.01 M citrate buffer, followed by drying at 100 °C for 1 h [1].

Sample application

Bandwise with Linomat 4, band length 5.0 mm, distance from left edge 10.0 mm, distance from lower edge 10.0 mm, application volumes 1 µL for standard solutions and 3 µL for sample solutions

Chromatography

Two-fold development with acetonitrile – water 4:1 in the Twin Trough Chamber 20 x 20 cm up to a migration distance of 75 mm from the lower plate edge, drying at room temperature for 5 min

Postchromatographic derivatization

The plates are immersed in the 4-aminobenzoic acid reagent with the Chromatogram Immersion Device (immersion speed 3 cm/s, immersion time 0 s) and heated in a drying oven for 15 min at 130 °C. Alternatively, the Derivatizer can be used for reagent transfer.

Densitometry

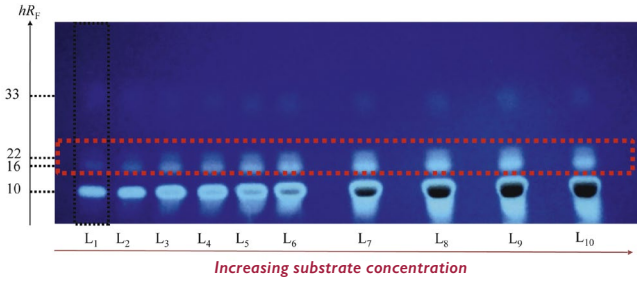
TLC Scanner 3 and winCATS 3.0, absorption measurement at 390 nm, slit dimension 6.00 x 0.45 mm, evaluation via peak area, polynomial regression

TLC-MALDI-TOF MS

The samples are applied in duplicate so that the plate can be cut after development into two halves, one for detection of sugars under UV 366 nm after derivatization, the other half for MALDI-TOF MS analysis. Each zone detected on the derivatized plate part is localized and the respective position marked on the non-derivatized half. After application of a magnetic nanoparticle matrix of core-shell silica-coated iron oxide, the zones are directly desorbed with a 337-nm nitrogen laser using the TLC-MALDI-Interface (5.0 cm x 7.5 cm) and Autoflex Mass Spectrometer (Bruker Daltonik).

Results and discussion

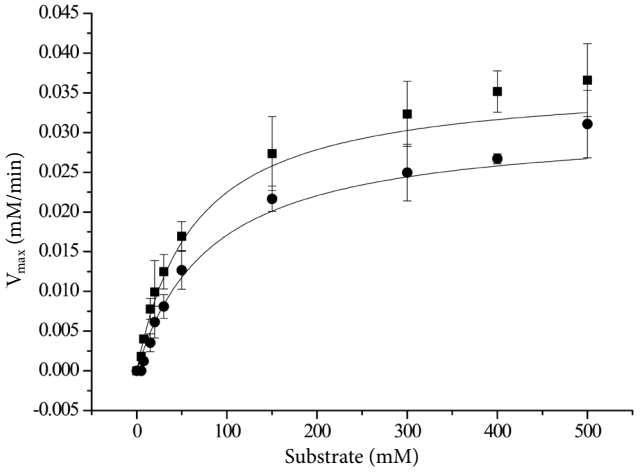
Densitometric screening was performed at 390 nm to detect substrates and products of the invertase reaction. All validation parameters (according to the International Conference on Harmonization guideline Q2 R1) were within the acceptance criteria and proved the method to be suited for quantification of glucose and fructose.



2

UV 366 nm chromatogram of BfrA-catalyzed hydrolysis products of sucrose (L1–L10: solutions after enzymatic reaction with increasing sucrose concentrations 5, 7.5, 15, 20, 30, 50, 150, 300, 400 and 500 mM); hR_F 10: sucrose, hR_F 16: glucose, hR_F 22: fructose, hR_F 33: (IS) rhamnose)

The TLC method was found highly suited to perform an invertase kinetic study due to the high robustness, specificity, repeatability (%*RSD* ≤ 10%), and low detection and quantification limits (LOD 20 ng/band and LOQ 60 ng/band).



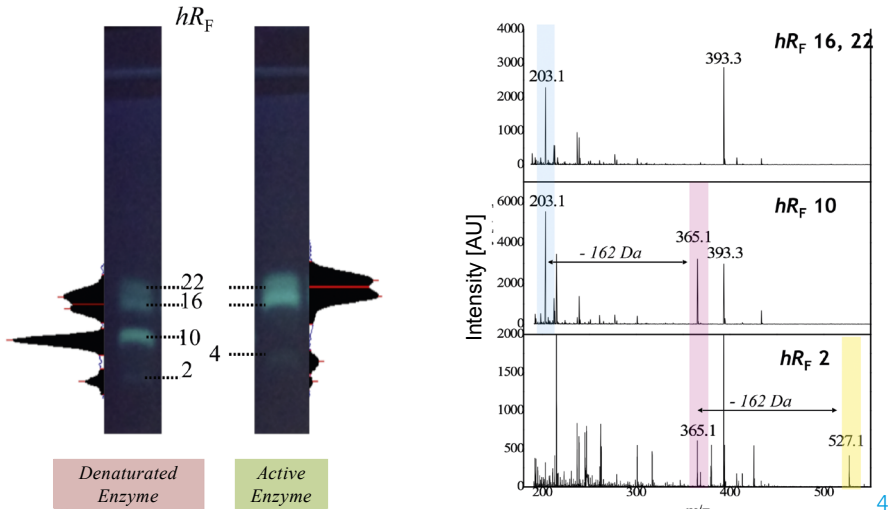
3

After sucrose-hydrolysis with BfrA, Michaelis-Menten plot of glucose (black squares) and fructose (black circles) obtained by absorption measurement at 390 nm

As expected, invertase hydrolyzes sucrose into an equimolar mixture of glucose and fructose. For both, similar kinetic parameters V_{max} and K_M were observed, close to those reported by other analytical techniques (UV, FTIR, and NMR) [3–5]. A specific fructose accumulation was observed for a higher polymerization degree and more complex sugar hydrolysis. This specific fructose-linkage recognized by invertase is present in vegetable saccharides, such as raffinose, stachyose and inulin.

Products	K_M (mM)	V_{max} (mM/min)	k_{cat} (s^{-1})	k_{cat}/K_M ($s^{-1}*mM^{-1}$)
Glucose	63.09 ± 7.60	0.037 ± 0.001	3.91	106.80
Fructose	83.01 ± 14.40	0.031 ± 0.002	3.30	106.50

MALDI-TOF-MS was used to identify and characterize the zones of interest [6]. Respective mass signals (sodium adducts $[M+Na]^+$) were obtained at *m/z* 527, 365 and 203. Sucrose (hR_F 10) and a trisaccharide (hR_F 2, potentially raffinose-hydrolyzed into melibiose and fructose) were detected in the plant extract.



UV 366 nm chromatogram and densitogram of a control (denaturated enzyme) and hydrolysis product (active enzyme) with plant extract LV 1784 as well as mass spectra of control zones (of non-derivatized plate)

Densitometry and MALDI-TOF MS have proven to be suitable detection tools for the identification of substrates and products of the invertase hydrolysis in plant extracts. TLC-UV-MS could be a valuable alternative to classical colorimetric tests, such as the 3,5-dinitrosalicylic acid (DNS), which cannot detect specific suitable substrates and products in complex mixtures.

[1] M. Ghebregzabher *et al.* J. Chromatogr. 127 (1976) 133–162

[2] J. Ferey *et al.* J. Chromatogr. A 1477 (2016) 108–113

[3] S. Belaz *et al.* Carbohydr. Res. 415 (2015) 31–38

[4] R. Schindler *et al.* Vib. Spectrosc. 16 (1998) 127–135

[5] J.D. Kehlbeck *et al.* J. Chem. Educ. 91 (2014) 734–738

[6] J. Ferey *et al.* Talanta 170 (2017) 419–424

Further information is available on request from the authors.

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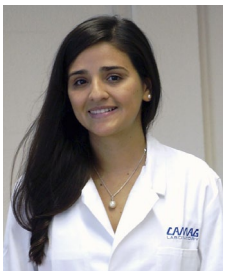
Comprehensive HPTLC fingerprinting for the quality control of *Angelica gigas* root



Débora A. Frommenwiler, CAMAG

Introduction

For the quality control of herbal drugs, pharmacopoeial monographs describe a suite of tests. They include verification of the identity and purity of a herbal drug as well as the determination of the amount of active substance(s) or analytical marker(s). For identification, HPTLC is generally the method of choice. If the software *visionCATS* is used to obtain HPTLC fingerprints, information beyond identity is readily available as well. While the term fingerprint usually refers to the electronic image of the visual HPTLC chromatogram, "comprehensive fingerprinting" includes the generation of a peak profile based on luminance, which can be evaluated quantitatively. Comprehensive fingerprinting thus provides information about the identity, purity, and minimum content of the herbal drug, all in a single analysis.

The case study of *Angelica gigas* roots [1] illustrates this concept. The roots of different *Angelica* species are traded in the Asian herbal market under the same common name Dang gui. *Angelica sinensis, A. acutiloba*, and *A. gigas* are the most commonly used species in China, Japan, and the Republic of Korea. They show similar phenotype and organoleptic properties, but differ in the chemical composition. Additionally, the traditional medicine systems of other regions prescribe many related species from the same plant family for similar purpose. Therefore a clear distinction is needed.

This work describes, as a proof of concept, how comprehensive HPTLC fingerprinting can be applied to control the quality of a herbal drug. A specific HPTLC method for identification was adapted. It was used to identify *Angelica gigas* and to distinguish 27 related species [1]. Additionally, the method was used to detect the presence of mixtures with two other *Angelica* species traded as Dang gui and further to test the minimum content of the sum of decursin and decursinol angelate in *Angelica gigas* root.

Standard solutions

System suitability test (SST): 1 mg of each imperatorin, osthole, and isoimperatorin are dissolved in 1 mL of methanol. Decursin standard solutions for quantification: five working solutions in concentrations between 8.0–40.0 µL/mL.

Sample preparation

1.0 g of the powdered root is extracted with 5 mL of methanol by shaking for 10 min. The suspension is centrifuged for 5 min. The amount of herbal reference material is adjusted to yield a solution representing a content of 3.0% of decursin and decursinol angelate (D+DA) on the dry basis. The supernatant is used for application. For the quantitative evaluation in the linear working range, the solutions are diluted 500-fold.

Chromatogram layer

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

Sample application

Automatic TLC Sampler (ATS 4), bandwise application, 15 tracks, band length 8.0 mm, distance from left edge 20.0 mm, distance from lower edge 8.0 mm, application volume 2 µL

Chromatography

In the ADC 2 with chamber saturation (with filter paper) for 20 min and after conditioning at 33% relative humidity for 10 min using a saturated solution of magnesium chloride, development with toluene – ethyl acetate – and acetic acid 90:10:1 to the migration distance of 70 mm (from the lower edge), drying for 5 min

Postchromatographic derivatization

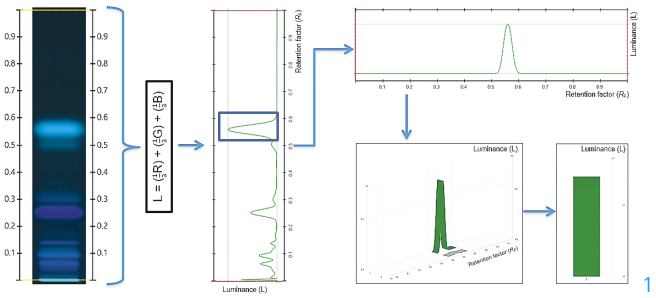
The plate is immersed into 10% sulfuric acid in methanol using the Chromatogram Immersion Device, immersion speed 5 cm/s and immersion time 1 s, and heated at 100 °C for 3 min using the TLC Plate Heater.

Documentation

TLC Visualizer under UV 254 and 366 nm (both prior to derivatization), UV 366 nm and white light (after derivatization).

Results and discussion

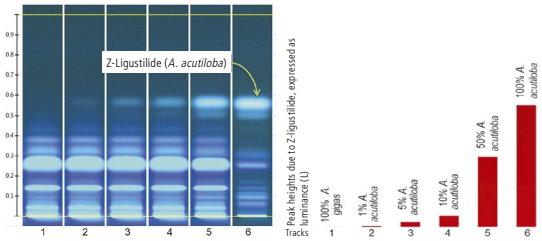
The HPTLC method for identification of *Angelica sinensis* root from the European Pharmacopoeia [2] was optimized and applied for the identification of *Angelica gigas* root and 27 related species. All species could be distinguished utilizing different detection modes, prior to and after derivatization. For the purity test (detection of mixtures with other species), the powdered drug *A. gigas* was mixed in different proportions with either *A. sinensis*, or *A. acutiloba*. *Z*-ligustilide is detected when only 1% of either *A. acutiloba* or *A. sinensis* is added to *A. gigas*. The zone due to *Z*-ligustilide is absent in *A. gigas*. The electronic images of the HPTLC fingerprints (HPTLC chromatograms) were converted into peak profiles and the peak heights of *Z*-ligustilide were compared side by side.



1

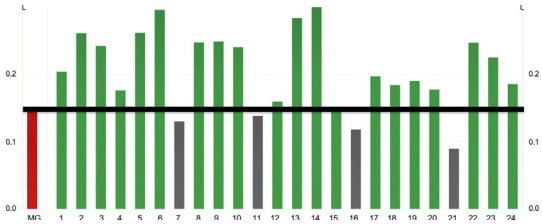
Conversion of an electronic HPTLC fingerprint into a peak profile

For the determination of the content, the zone at hR_F 27 was chosen due to the co-eluting markers decursin and decursinol angelate (D+DA). As reference material, a sample of *A. gigas* root with a known content of D+DA was used. It was prepared to represent a drug with the content of 3.0% D+DA. The fingerprints under UV 366 nm prior to derivatization were converted into peak profiles and the peak heights of D+DA were compared. 4 of 24 samples failed the test for minimum content.



2

Comparison of Z-ligustilide peak heights from image profiles of HPTLC fingerprints of A. gigas mixed with A. acutiloba; left: HPTLC chromatograms under UV 366 nm prior to derivatization (contrast 1.0; normalized over Z-ligustilide zone of track 5)



3

Comparison of the content of 24 different samples with the minimum content (MC) [1].

The method was validated for overall performance in a collaborative trial involving three laboratories, including the laboratories of the national authorities of Korea and Vietnam. In this study, based on the electronic image of the HPTLC fingerprint, the specifications for the chemical identification of *Angelica gigas* roots, the determination of the purity (detection of adulteration with *A. sinensis* and *A. acutiloba*), and the content of D+DA were established. The concept of comprehensive HPTLC fingerprinting proved to be an efficient, pragmatic, and cost-efficient approach for establishing specifications and controlling the quality of an herbal drug.

[1] D. Frommenwiler *et al.*, Planta Medica 84 (2018) 465–474

[2] European Pharmacopoeia, 9th Edition. Monographs for *Angelica sinensis* root (2558). Strasbourg: European Directorate for the Quality of Medicine and Healthcare (EDQM), 2017, 1242–1248

Further information is available on request from the author.

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Fast determination of benzoic acid in food

From left: Michaela Oberle, Petra Lewits (both Merck) und Falk-Thilo Ferse (Waters)

Introduction

Preservatives are becoming more and more important in food production and thus in analytics. One of the most important preservatives for the food industry is benzoic acid [E 210]. It is used to extend the shelf life of products like sausages, canned fish, spirits and cosmetics by preventing the growth of fungi and bacteria.

Benzoic acid is believed to cause several allergic reactions such as asthma and hives. Therefore, their analysis is of great importance.

The analysis of complex samples, such as food, is often associated with a labor-intensive sample preparation. The following workflow describes a fast, and at the same time very precise, method for the determination and identification of benzoic acid in crabs and smoked salmon without a time-consuming additional sample preparation. It is based on the combination of HPTLC with UV absorption measurement and mass spectrometry. By UV absorption measurement with the TLC Scanner, the content of benzoic acid in many food samples can be determined in parallel and identity of benzoic acid zones can be confirmed by mass spectrometry.

Standard solution

10 mg benzoic acid in 10 mL methanol

Sample preparation

125 g crab or 75 g salmon are homogenized in 100 mL of water with an Ultra-Turrax and filtered.

Chromatogram layer

HPTLC Silica gel 60 CN F_{254S} (Merck), 10 x 10 cm

Sample application

Bandwise with Automatic TLC Sampler (ATS 4), band length 6.0 mm, 10 tracks, distance from left edge 15.0 mm, distance from lower edge 10.0 mm. Application volume 0.1–2.0 µL for standard solutions and 0.4–0.6 µL for sample solutions

Chromatography

In a Twin Trough Chamber 10 x 10 cm, with ethanol – water 1:4 + 0.01% *n*-heptane to the migration distance of 60 mm (from the lower edge), drying for 10 min at 60 °C on the TLC Plate Heater

Postchromatographic derivatization

The plate is sprayed with anisaldehyde-sulfuric acid-reagent using the Derivatizer and heated at 120 °C for 10 min using the TLC Plate Heater.

Documentation

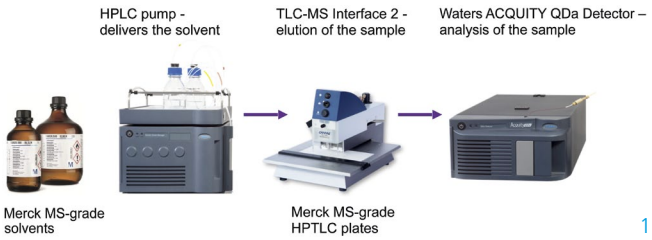
TLC Visualizer under 254 nm, 366 nm, and white light

Densitometry

TLC Scanner 3 and winCATS, absorption measurement at 232 nm, slit dimension 4.00 x 0.20 mm, scanning speed 50 mm/s

Mass spectrometry

Elution of zones with TLC-MS Interface (oval elution head) at a flow rate of 0.1 mL/min with acetonitrile + 0.1% ammonia into an ESI-MS (ACQUITY QDa, Waters) and detected in negative ionization mode (*m/z* 50–600).

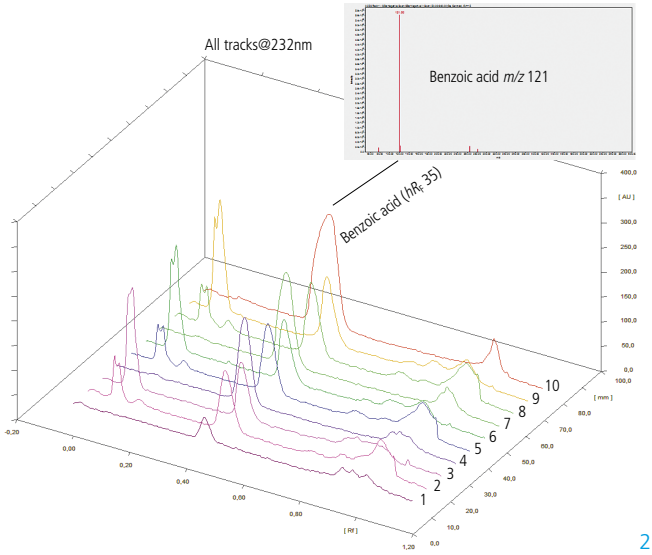


1

Elution of a sample for confirmation by mass spectrometry

Results and discussion

With this HPTLC-UV method 0.65 mg/g benzoic acid could be quantified in the analyzed crab sample and 0.54 mg/g in the salmon sample. The correlation coefficient r^2 was 0.9996, the relative standard deviation 3.6%. HPTLC-MS was used for confirmation of the obtained results. The signal of the deprotonated molecule was at *m/z* 121 $[M-H]^-$. In addition, the food dyes cochenille red [E 124] and yellow orange S [E 110] were found in the salmon sample.



2

Densitograms of the standard (tracks 1, 4, 7, 10), the crab sample (tracks 2, 5, 8) and the salmon sample (tracks 3, 6, 9) and mass spectrum of the benzoic acid standard (m/z 121 $[M-H]^-$)

Further information is available on request from the authors.

Contact: Michaela Oberle, Merck KGaA, Frankfurter Str. 250, 64293 Darmstadt, Germany, michaela.oberle@merckgroup.com

3

CAMAG TLC-MS Interface 2

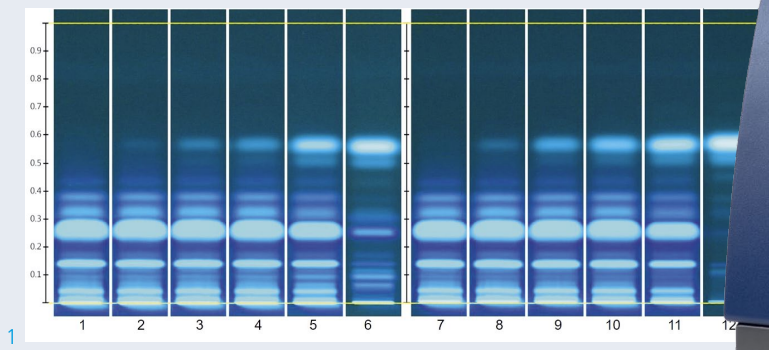
The CAMAG TLC-MS Interface 2 is the second generation of our module for the pioneering concept of hyphenating HPTLC with mass spectrometry. Plate positioning is significantly simplified. The elution head has been modified and an easily accessible, exchangeable filter has been arranged in front of the valve.

Cleaning is facilitated as compared to the previous version, making it highly efficient. By pushing a button, the elution path is cleaned of matrix particles with compressed air, increasing the lifetime of the filter and preventing the system from becoming blocked. Filters are separately available and can be easily replaced without any modification to the elution head.

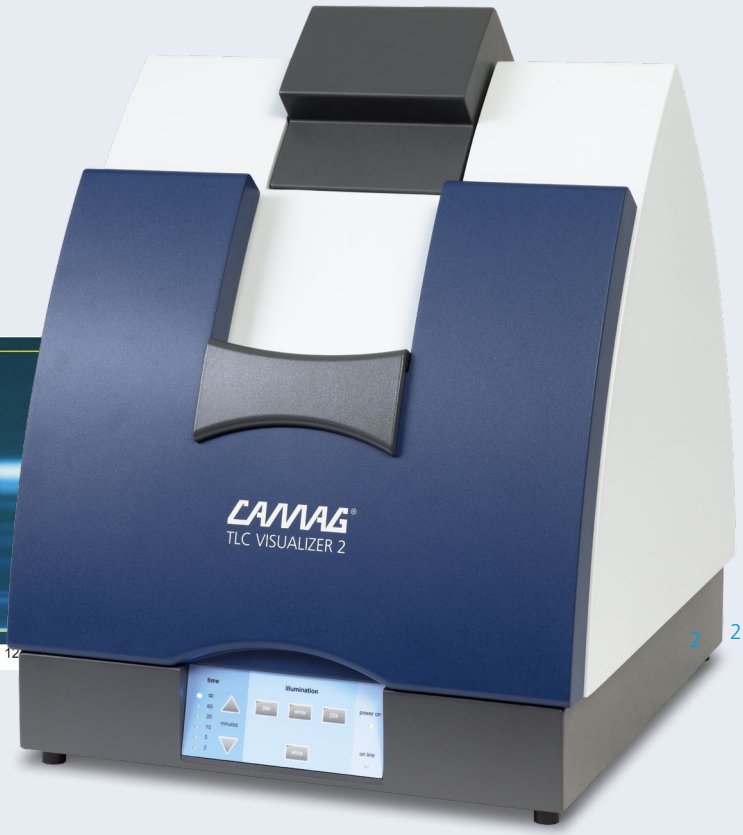
The CAMAG TLC-MS Interface 2 allows for rapid and contamination-free elution of TLC/HPTLC zones with online transfer to a mass spectrometer. The interface can be installed plug & play in any LC-MS system without adjustments or mass spectrometer modifications. Depending on the MS system, the presence of a substance can be confirmed via its mass spectrum, or for an unknown substance, the respective sum formula is obtained within a minute.

CAMAG TLC Visualizer 2

Professional imaging and documentation system for TLC/HPTLC chromatograms



HPTLC Chromatogram of Angelica acutiloba and Angelica sinensis under UV 366 nm (CBS 121 pp. 12–13)



The TLC Visualizer 2 is equipped with more technical innovations, offering better image quality than its predecessor:

- Digital CCD camera with a maximum resolution of 82 µm on the plate
- USB 3.0 for easy PC connection and rapid data transfer
- Simple operation under *visionCATS* (from version 2.3 on)

Further information
www.camag.com/tlcvisualizer2

With this high-end imaging system, reproducible images of superior quality are obtained under different illumination modes – white light, UV 366 nm, and UV 254 nm. The software controlled operation offers:

- Image enhancement tools – Spot Amplification, Clean Plate Correction and Exposure Normalization
- Evaluation possibilities – Quantitative evaluation of image profiles and "Comparison Viewer" of tracks from different plates and/or different illumination modes

The TLC Visualizer 2 meets all requirements to be utilized in a cGMP/cGLP environment.

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CBS 121 E 996.2270-24