CBS 113 • SEPTEMBER 2014

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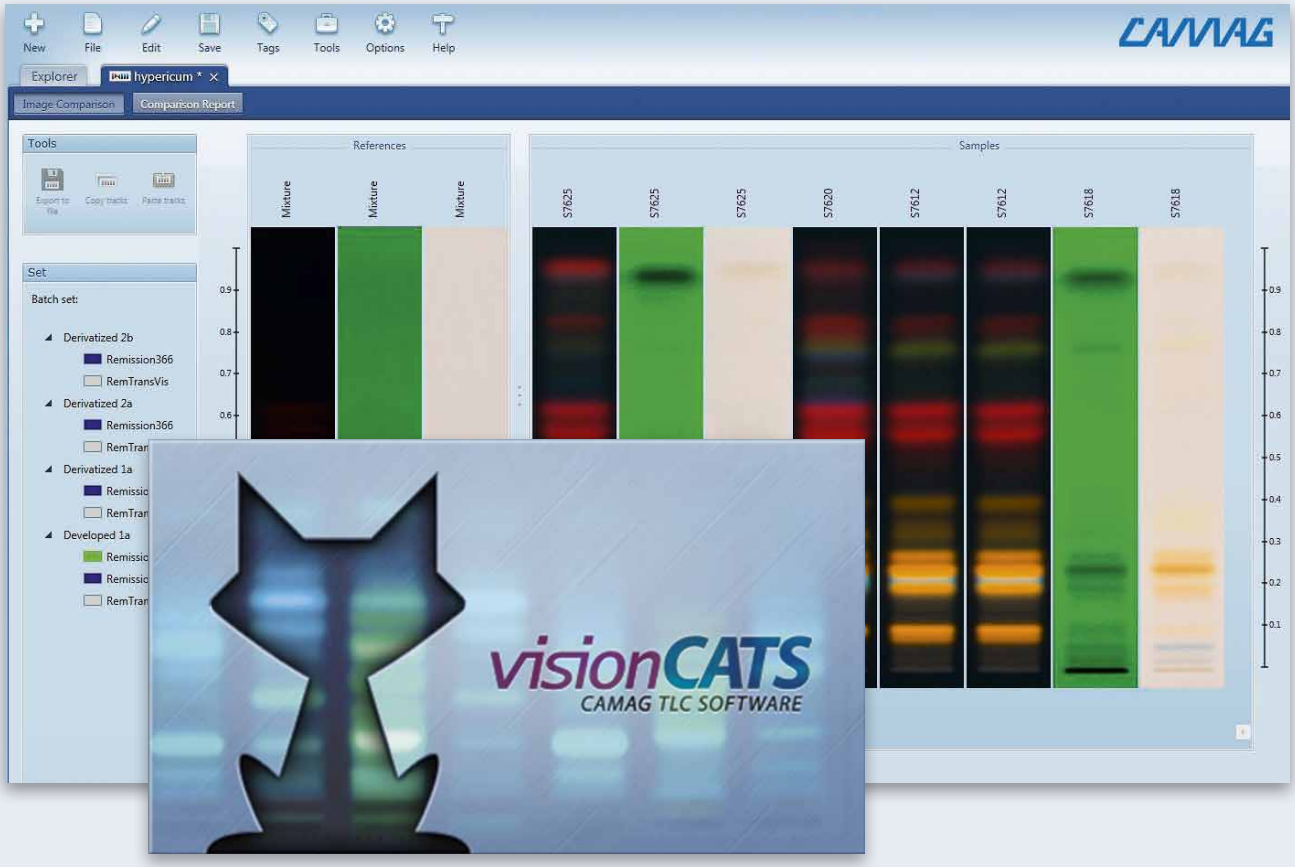
113

SEPTEMBER 2014



Introducing the new generation HPTLC software

Other topics of this issue:
Analysis of drinking water – caffeine in energy drinks – tyrosinase inhibitors – plant glycosylceramides

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IN THIS ISSUE

Procedures, applications

Products featured in this issue

Column: Know CAMAG

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Introducing the new generation software – visionCATS

CAMAG is in the final stage of developing the new generation software visionCATS. While developing we incorporated more customer feedback than in any previous CAMAG software and fine-tuned our designs with user tests. New features have been included that meet the needs of cGMP compliant routine work, while leaving the flexibility for method development in scientific research.

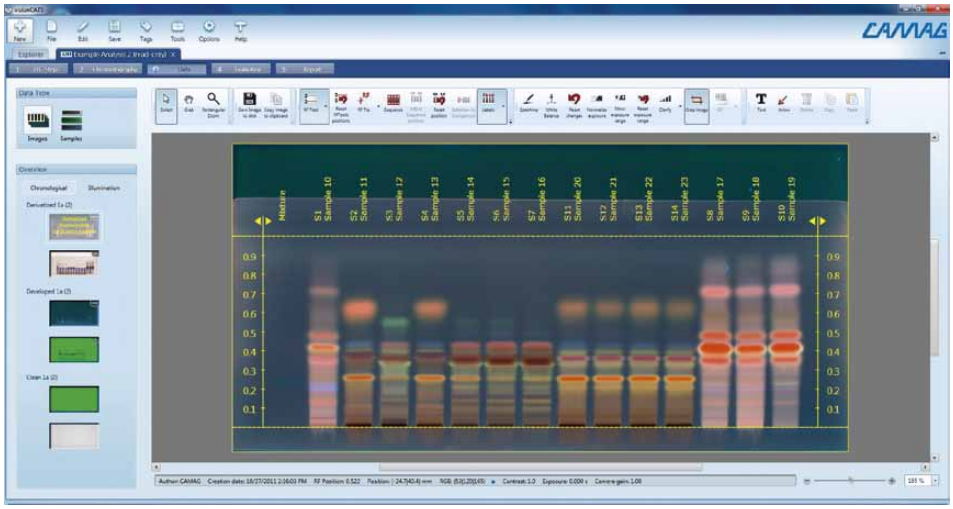
In its present stage visionCATS is able to control CAMAG's following instruments, the Linomat 5, the Automatic TLC Sampler ATS 4, the Automatic Developing Chamber ADC 2 and the TLC Visualizer documentation and evaluation system, in other words all the instruments needed to perform qualitative HPTLC analysis. Its new features are so convincing that we decided to not wait and deprive our customers of them but release this part of visionCATS. You can expect the same excellence from the quantitative version which we plan to release in early 2015.

Based on a state-of-the-art three-tier architecture visionCATS can now run on a multi-user lab network. Image comparison between sample tracks acquired at any detection mode from different plates is now possible. The sample-based approach allows to evaluate samples and references within a comparison file from which individual reports can be generated by a mouse click. New image enhancement tools ensure the best image quality for identification of even the weakest zones. The background noise is minimized by the "clean plate" image correction. It provides together with a standardized methodology, validated methods and reference images, the basis for a plug & play approach to HPTLC: After installation of instruments followed by qualification, a validated method is uploaded (plug) and the user is guided through the analysis (play).

Key features of visionCATS

- **Intuitive handling – HPTLC has never been as easy**

  A modern clearly structured graphic user interface puts the user in complete control of the HPTLC process. Whether it is managing of CAMAG instruments or acquiring, storing/retrieving, and evaluating of analytical data, all tasks can be performed with a few mouse-clicks. The menus are self-explanatory but in case of questions, help files and tutorials give answers. Analyses in a routine setting can be performed based on validated methods available within the software. visionCATS guides the user through all steps of the chromatographic process. The only required input is to fill in the sequence table with the sample's ID and to set the position on the plate onto which the sample is to be applied. If in a research setting, flexibility of the analytical work is of interest, all parameters of the HPTLC process are accessible for change.



1

Data view of an analysis

- **Client server system – access to the same data for all members of a work group**

  visionCATS uses a state-of-the-art software architecture. A client server system offers enormous flexibility to the number of instruments and users that are working together. In the simplest case of a small laboratory, client and server as well as all available instruments are installed/connected to the same computer. In larger organizations instruments in different laboratories can be networked to the same server so that users in different locations can work with the software through multiple clients (individual workstations).

2

Image comparison of individual tracks in different detection modes

- **Database and comparison tool – unleash the power of HPTLC**

  Unlike other TLC software on the market visionCATS features a powerful database at its heart. While samples are still analyzed in the context of a plate it is now possible to retrieve all data associated with each individual sample based on the sample ID. A visionary comparison tool has the potential

of changing the way the analytical world looks at HPTLC analysis: Data (fingerprints) of multiple samples or detection modes can be viewed side by side. Samples can be re-arranged and compared to reference substances or samples and it is irrelevant from which plate the data comes, as long as it has been qualified by a system suitability test. Thus something like "artificial" plates can be generated that include dozens of tracks. True collaboration between laboratories is no longer a wishful thinking: method files, analysis files (data pertaining to a plate) and comparison files can be exported by one user and imported into another user's database. That means unique or rare samples/standards do not have to be sent around. Their chromatograms can be used as reference images for comparison.

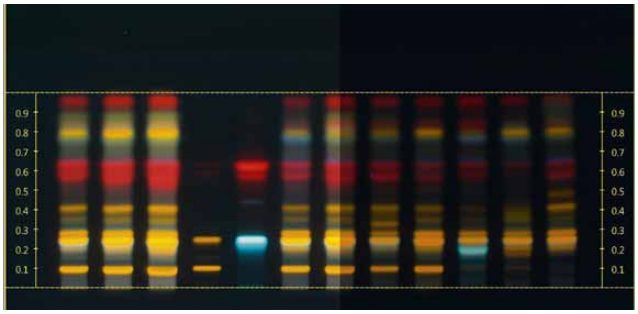
- **HPTLC method library – convenient download of validated methods**

  For analysts using HPTLC for identification of plants visionCATS offers break through support with a Method Library for seamless import of validated methods. Licensed users can download library files and import them into their own database. Each file includes:

  - A method document (.docx) which serves as an SOP and can easily be incorporated into any quality management system.
  - An instrument method which is ready for execution. A new analysis with all method relevant information (reference substances, plate layout, stationary and mobile phase, etc.) can then be processed. Only the vial ID of the samples to be analyzed needs to be entered.
  - A comparison file allows to compare each individual sample with images of chemical and botanical reference materials and to evaluate the results based on acceptance criteria set forth in the method document. After comparing the data of the samples with the reference images individual reports can be generated by a mouse click.
  - Currently the method library is being populated with all methods included in the USP Dietary Supplement Compendium and those published by the International Association for the Advancement of HPTLC. Numerous methods of identification from the European Pharmacopoeia are added as well.

What else is new?

Since its release as a beta version visionCATS has been continuously improved for usability with the help of customer feedback. The current version 1.4 is clearly focused on qualitative analysis based on digital images and unlocks the full potential of the CAMAG Visualizer. Low noise, high dynamic range images and the new tool "exposure normalization" allow comparison of samples from different plate with virtually the same exposure settings. The "clarify" tool can visualize very faint zones on an unchanged background.



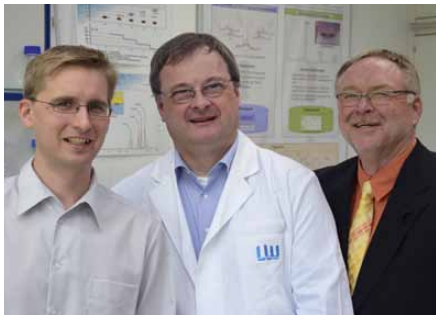
3

Amplified image for visualization of weak zones (left) and original image (right)

To conclude, visionCATS is a server-based application to control, perform and document HPTLC analyses in a multi-user lab network. In addition to these qualitative possibilities, the quantitative version, including quantitative evaluation by scanning densitometry, will follow soon. Look for the latest information in CBS 114.

Download a fully functional version for a 60 day trial and see how visionCATS can change your appreciation of HPTLC.

HPTLC-MS combined with H/D exchange for the identification of substances in environmental analysis



From left: Stefan C. Weiss, Wolfgang Schulz, Walter H. Weber *Alexander Müller*

In order to ensure the high quality of the drinking water, the laboratory for operation control and research of Zweckverband Landeswasserversorgung in Langenau, Germany, uses an array of modern analytical methods to search for traces of organic substances in raw, processed and drinking water. The aim of these analyses is to identify the infiltration of substances in raw water resources and to describe their origin. This is a prerequisite to reduce contamination.

Introduction

During the restoration of a landfill the laboratory was contracted for analytical services. HPTLC/AMD was one of the analytical solutions used as a screening method to identify previously unconsidered substances. The separated substances were eluted with the TLC-MS Interface and analyzed with a high resolution mass spectrometer (QTOF/MS). Using the exact mass and the isotopic pattern, it was possible to assign a molecular formula to the unknown compounds. With the molecular formula information, queries in different databases (*e.g.* DAIOS-online, Pubchem and ChemSpider) for known substances were performed. Depending on the molecular formula this can yield up to a few thousand possible structures. To reduce the quantity of possible structures, H/D exchange may be used. Via elution with D_2O almost all exchangeable protons in the molecule are replaced by deuterium. Every hydrogen in the molecular ion that is replaced by deuterium leads to a mass shift of 1.0063 Da in the ESI mass spectra.

HPLC has only limited suitability for this propose because the chromatography has to be done in expensive deuterated solvents [1]. An alternative is to perform the H/D exchange after the HPLC separation in the electrospray ("on the fly") [2]. But by doing so, a complete exchange of all exchangeable protons cannot be guaranteed.

A better approach is to realize the H/D exchange after chromatography by coupling HPTLC and MS, because between separation and transfer of the substance into MS a solvent change occurs. For this reason only a few milliliters of D_2O is needed for an almost complete exchange of all exchangeable protons by elution with the TLC-MS Interface.

Sample preparation

The analyte was enriched from a 1 L water sample by solid phase extraction (SPE, 0.2 g of Isolute ENV+) at pH 3 or 7, respectively. The SPE sorbent was conditioned consecutively with 6 mL of each solvent, *n*-hexane, acetone, methanol and water (pH 3 or 7, respectively). After drying 6 mL of methanol were used for elution. After careful evaporation of the solvent under a stream of nitrogen at 40 °C to near dryness the residue was taken up in 200 µL of methanol.

Layer

HPTLC plates LiChrospher F_{254} (Merck) 20 x 10 cm were prewashed with 2-propanol, dried at 120 °C for 20 min with the TLC Plate Heater, predeveloped with acetonitrile and dried again at 120 °C for 20 min.

Sample application

Samples were applied as area of 6 x 3 mm with a track distance of 12 mm with the Automatic TLC Sampler 4 (ATS 4).

Chromatography

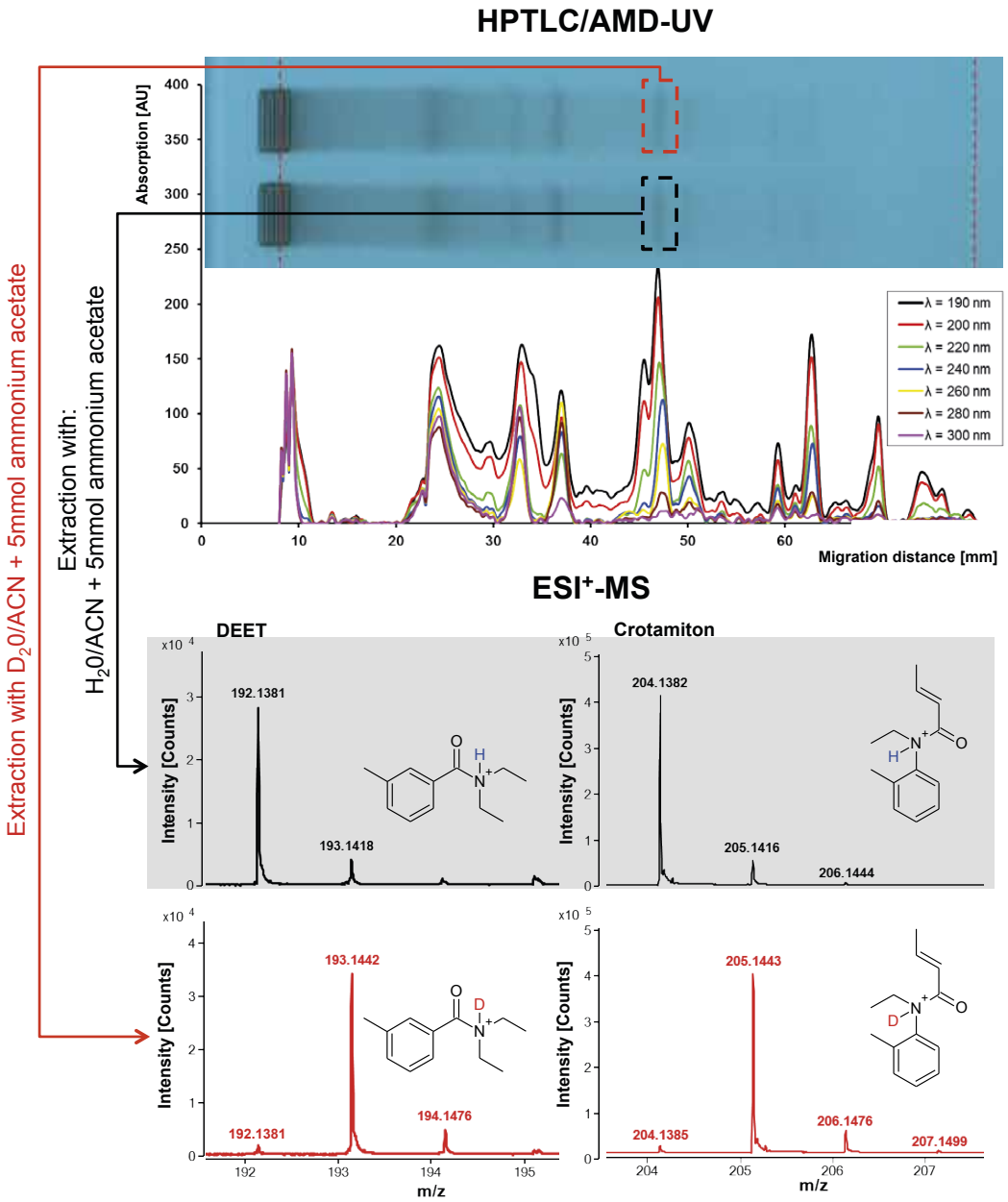
In the AMD 2 system with a 25-step gradient, starting in the isocratic mode with 5 steps acetonitrile – dichloromethane 1:1 for focusing, followed by 15 steps from acetonitrile – dichloromethane 1:1 to dichloromethane and then 5 steps from dichloromethane – *n*-hexane 4:1 to *n*-hexane. The final migration distance was 80 mm.

Densitometry

Multiple wavelengths scan at 190, 200, 220, 240, 260, 280, 300 nm with the TLC Scanner 3 and winCATS after documentation under UV 254 nm, UV 366 nm and white light illumination.

HPTLC-MS

Coupling of HPTLC with QTOF/MS (6520, Agilent Technologies) using the TLC-MS Interface offline with water – acetonitrile 1:1 and deuterium oxide – acetonitrile 1:1, respectively, each with an addition of 5 mmol/L ammonium acetate, at a flow rate of 0.2 mL/min using the oval elution head (4 x 2 mm).



Identification of DEET and crotamiton a landfill leachate by investigation on exchangeable protons via HPTLC-MS 1

Results and discussion

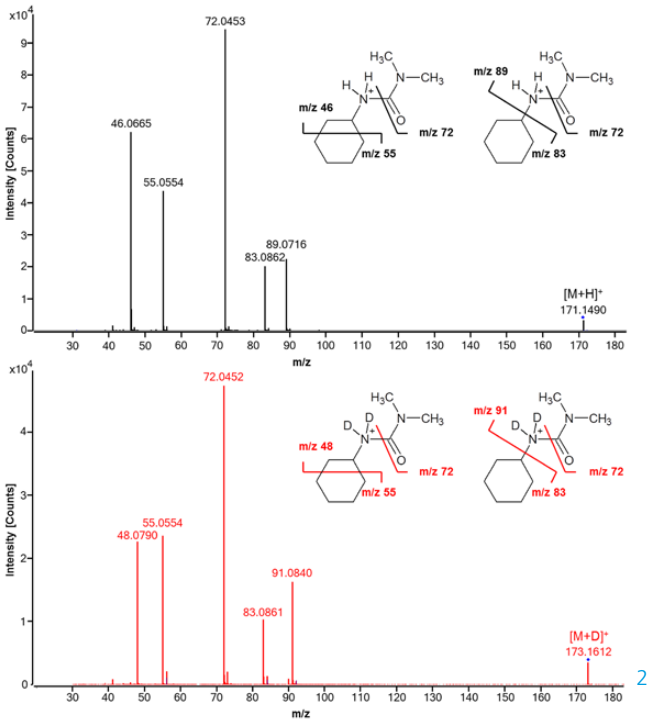
The SPE extracts were separated with the described AMD 2 gradient for the identification of substances from the landfill leachate. The multiple wavelengths scan of the sample from the landfill leachate showed a peak at 47 mm. The zones of the first track were eluted with water/acetonitrile with 5 mmol/L ammonium acetate.

It was possible to detect two compounds with *m/z* 192.1381 and 204.1382 in the water/acetonitrile eluate using the positive electrospray ionisation mode (ESI^+). Together with the isotopic pattern the molecular formula could be assigned as $C_{12}H_{17}NO$ and $C_{13}H_{17}NO$, respectively. A mass shift of only 1,006 Da was observed in the mass spectra for both compounds. It is concluded that there are no further exchangeable protons in the molecule as both compounds were detected as their correspondent quasi molecular ions in the ESI^+ mass spectrum $[M+H]^+$ and $[M+D]^+$, respectively.

The chemspider database query for $C_{12}H_{17}NO$ yielded 2652 possible structures. By limiting the hits to only substance with metadata available (*e.g.* patents, spectral data, etc.) the possible structures were reduced to 58. With the information from the H/D exchange experiment the database hits were reduced again to 22. Among these remaining hits were phendimetrazine and N,N-diethyl-*m*-toluamide (DEET). The insect repellent DEET could be identified to be compound $C_{12}H_{17}NO$ using reference standards and the molecular formula $C_{13}H_{17}NO$ could be assigned to the pharmaceutical crotamiton.

In another ground water sample a conspicuous band was also detected. The zone was also eluted on the first track with water/acetonitrile and on the other one with D_2O/acetonitrile in order to identify the compounds. The MS analysis of both eluates showed that the substance with a molecular formula of $C_9H_{18}N_2O$ possesses one exchangeable proton. For structure elucidation, MS/MS experiments were done from both eluates. With the help of the MS/MS spectra the compound 3-cyclohexyl-1,1-dimethylurea is assigned to the conspicuous band.

The described analytical procedure represents a generally applicable method, supporting identification of substances. It could be demonstrated that for the elution of substances from the chromatogram only a few milliliters of D_2O were needed to achieve an almost complete exchange of all exchangeable protons. The knowledge of exchangeable protons in a molecule helps to reduce the number of possible structures. The H/D exchange was also useful for the interpretation of MS/MS spectra. With other specific derivatizations the H/D exchange could also be a helpful tool for the identification of substances after HPTLC separation.



2

Comparison of the MS/MS spectra of 3-cyclohexyl-1,1-dimethylurea $[M+H]^+$ and deuterated 3-cyclohexyl-1,1-dimethylurea $[M+D]^+$

[1] D. Q. Liu *et. al.*, Rapid Commun Mass Spectrom 15 (2001) 1832

[2] J.-C. Wolff, A. M.-F. Laures, Rapid Commun. Mass Spectrom 20 (2006) 3769

Further information is available from the authors on request.

Contact: Stefan C. Weiss, Zweckverband Landeswasserversorgung, Betriebs- und Forschungslaboratorium, Am Spitzigen Berg 1, 89129 Langenau, Germany, weiss.s@lw-online.de

New face in the Marketing Department

Aydin Mohadjer (33)

In November 2012 I succeeded Lukas Frommenwiler as Marketing Manager. Following my business studies in Heidelberg, Germany with a focus on Marketing, I joined a Zurich-based, Swiss software company as the Assistant Marketing Manager.

After a few years at this company, I decided that I would like to live in Basel. I soon joined CAMAG, which I saw as a modern and customer-oriented company with a broad range of marketing activities, in my mind a good opportunity for someone with my background.

To support our customers we plan to be more targeted in answering their analytical questions and meeting their needs. We propose to present case studies on the various application areas of HPTLC, thus illustrating the unique characteristics of planar chromatography. Application videos will graphically show the practical steps of each HPTLC case study and introduce the required instruments for performing the respective standardized method. The first case study is already available online, more to follow soon.

Another important innovation is the online version of the database "Cumulative CAMAG Bibliography Service CCBS". Research can now be performed online without downloading the entire database. For more information, please see page 3 of the Yellow Pages in this CBS issue.

Be sure to take a look at the first case study and feel free to nominate others.

Aydin Mohadjer
Marketing Manager

CBS 113
CAMAG BIBLIOGRAPHY SERVICE
SEPTEMBER 2014

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

From this CBS the abstracts of 128 papers have been added to the CCBS database. Several of these deal with digital evaluation of chromatograms, either focused on office scanners or digital image analysis. Comparable results were reported for image analysis, when compared to conventional evaluation by a TLC scanner. However, for the latter, the LOD/LOQ was better, as it allowed measurements at the maximal absorption wavelength. On goingly more validation data are being reported for the HPTLC methods being used, which underlines the increasing use of quantitative HPTLC. In several abstracts LOD/LOQ could have been improved by using more sensitive reagents, *e.g.* immersion into orcinol solution for sugar analysis is not as sensitive as dipping into aniline diphenylamine *o*-phosphoric acid reagent or ß-naphthol reagent or *p*-aminobenzoic acid reagent. Also, exposure to iodine vapors for derivatization and quantitation might not be the best combination.

Despite this positive trend towards reporting of reliable HPTLC methods, still many TLC/HPTLC abstracts on TCM analyses report the use of TLC/HPTLC followed by quantification of the active compound by HPLC. There is no need for an extra method if quantitative HPTLC is used, with or without selective derivatization. Many standards subjected to the extra HPLC method have already been quantitatively detected in other HPTLC studies. So it seems that skilled training in HPTLC is required to break off a traditional, unnecessary and ineffective workflow. If there is the feeling that resolution is insufficient or you feel uncomfortable with optimization, feel free to contact the CAMAG laboratory.

Dear friends

Following the tendency to "Green Office" and online search, we have decided to no longer distribute the abstracts with information on TLC/HPTLC literature in printed form (Yellow Pages) and to make online access via internet more comfortable. From now on, the Cumulative CAMAG Bibliography Service (CCBS) will be available online for direct search within more than 11'000 abstracts of TLC/HPTLC publications. Via keyword search, you can generate your tailor-made research file, and only when necessary, print this selection. For example, if you search for CBS 113, you will find all the 128 abstracts added newly to the CCBS database with this CBS edition. This way, you do not miss the electronic yellow pages which were some of the most downloaded files on the CAMAG homepage. The handling of the new online database is described in detail on yellow page 3.

The recently held International Symposium on High-Performance Thin-Layer Chromatography, 2–4th July 2014, in Lyon, France, was a great success. A summary is presented on the last yellow page. We are already looking forward to the next Symposium, which is scheduled for beginning of July 2017 in Berlin, Germany.

Kind regards

Gertrud Morlock
cbs@camag.com

CAMAG LITERATURDIENST
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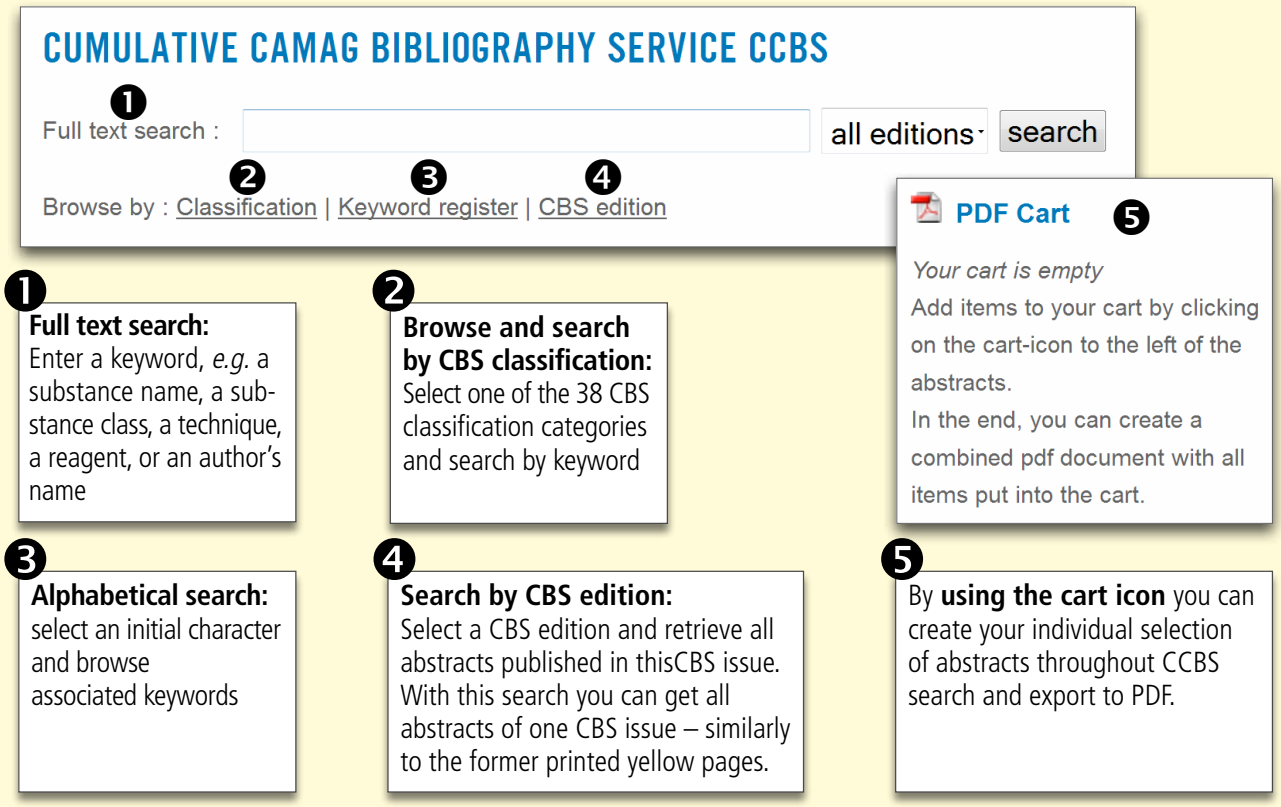
PLANAR CHROMATOGRAPHY

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**

XX. (abstract number underlined) refers to HPTLC related publication or application using HPTLC materials

New: Now you can directly search online the Cumulative CAMAG Bibliography Service (CCBS) database



The Cumulative CAMAG Bibliography Service (CCBS) is now available online without the need of downloading the entire database as has been the case in the past. This new online application will be updated after the publication of each new CBS edition. Currently, this extensive database includes more than 11'000 abstracts of publications between 1983 and today. 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. The printed abstracts will no longer be published in the yellow page format beginning with CBS 113 onwards.

CCBS covers numerous scientific journals, such as Journal of Chromatography A and B, Journal of Liquid Chromatography & Related Technologies, Journal of AOAC International, Journal of Planar Chromatography, Planta Medica, Analytical Letters, Food Chemistry, Trends in Analytical Chemistry, Journal of Ethnopharmacology, Tetrahedron, Rapid Communications in Mass Spectrometry, Journal of Pharmaceutical and Biomedical Analysis, Journal of Agricultural Food Chemistry, and Journal of Separation Science. In addition various publications in German, French, Spanish, Portuguese and Chinese are reviewed for CCBS.

With the Cumulative CAMAG Bibliography Service CCBS you can now perform your own detailed TLC/HPTLC online literature search.

Register now and explore the new CCBS Online Application. This service is free of charge for you.

www.camag.com/ccbs

International Symposium on High-Performance Thin-Layer Chromatography in Lyon

The recently held International Symposium on High-Performance Thin-Layer Chromatography in Lyon, France, 02–04, July 2014, was a great success and attracted scientists from about 25 countries, with leading participations from France, Germany, Poland, Switzerland, India, USA and Spain. All the nations enjoyed the unique charming French hospitality and great thank is owed to the organization committee! The auditorium was gender-balanced with equal representation from industry and academia. 190 Abstracts were submitted and actually 46 oral presentations were held and 108 posters presented. Practical workshops were followed by three comprehensive tutorials, a freshened, but fruitful panel discussion on research & development with representatives of five manufacturers, a historic lecture on Lyon and many other excellent presentations.

Some of the latest research was presented for the first time at the Symposium. Examples are new generations of miniaturized nanostructured or electrospun layer materials or substantial improvements in effect-directed analysis, *e.g.*, for sensitive detection of estrogens in complex samples down to the femtogram-per-zone range. Modern direct bioautography, generating sharp zones, seems to become an attractive tool for non-target analyses.

But also the effectiveness of HPTLC was proven by benchmarking with HPLC as shown for the example of the streamlined analysis of sugars in chicory root juice. The increased use of HPTLC-MS

was evident and new ionization sources were presented like the combined laser desorption/electrospray and atmospheric pressure chemical ionization source. Particularly young researchers presented their work.

The high quality throughout all the presentations was highly recognized: "I very much enjoyed the meeting as I was really impressed by quality of the work presented and the power of modern HPTLC" or "The technical quality of the meeting was extremely high with a large number of really excellent presentations". These are only some voices out of a plenty of positive feedbacks. So thanks to all the committed presenters – this is your great success!

All the poster presentations were greatly appreciated and provided a fertile ground for intense discussion, followed by collaborations. Knowledge was shared and new ideas discussed. This was also valued by many feedbacks: "Are the HPTLC meetings always this much fun and exchange of knowledge?" or "…really an excellent platform for learning and sharing" or "We had wanted to meet you for some time. The HPTLC symposium provided the perfect opportunity" or simply "It was really a piece of a very good work".

We are already looking forward to the next International HPTLC Symposium, scheduled in Berlin, Germany, in beginning of July 2017. Try to be better by keeping yourself updated and joining us!

HPTLC-UV/MS of caffeine in energy drinks

Introduction

In addition to the possibility to analyze many samples in parallel on one plate, its high matrix tolerance is a major strength of planar chromatography. Samples can be analyzed often without or with only a very simple sample preparation step. In combination with mass spectrometry very significant analytical information can be realized. To exploit the full potential of mass spectrometry, special MS-grade HPTLC plates offering high sensitivity and low background noise are now available.

HPTLC-UV/MS analysis of caffeine in energy drinks was performed directly without sample preparation on HPTLC plates silica gel 60 F_{254} MS-grade. Quantitation was performed by UV densitometry and confirmation by MS.

Layer

HPTLC plates silica gel 60 F_{254} MS-grade

Sample application

With Automatic TLC sampler 4 (ATS 4) 0.5, 1, 2 and 3 µL of the caffeine standard solution (100 ng/µL) as well as 0.5 µL of four energy drinks were applied.

Chromatography

Flat bottom chamber with 2-propanol – *n*-heptane – water 7:3:1

Derivatization

Staining with anisaldehyde-sulfuric-acid-reagent

Documentation and densitometry

With DigiStore 2 at UV 254 nm; scanning by absorbance with TLC Scanner 3 at 273 nm

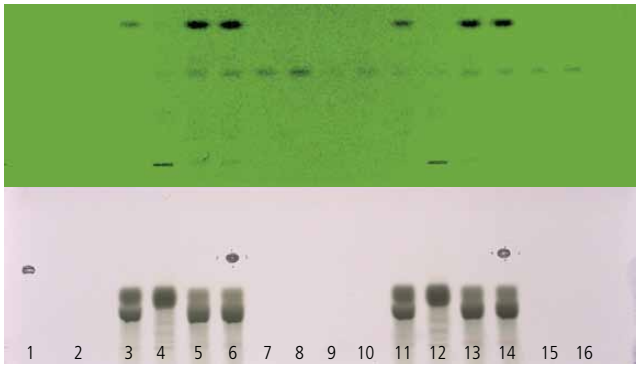
Mass spectrometry

The zones were eluted with the TLC-MS Interface (acetonitrile – water 95:5 with 0.1 % formic acid, flow rate 0.1 mL/min) and directly transferred to the expression CMS mass spectrometer (Advion) in ESI(+) full scan mode (*m/z* 100–500).

Results and discussion

The documentation at UV 254 nm clearly shows the caffeine zone (hR_F 55) without visible influence from matrix components, despite high matrix load being apparent in the sample tracks after derivatization.

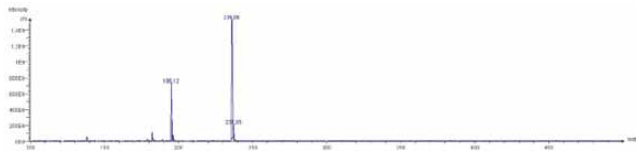
The quantitation of the four energy drinks resulted in caffeine contents between 130 and 410 mg/L. Two of the four energy drinks were above the maximum caffeine content of 320 mg/L for imported energy drinks in Germany.



1

HPTLC chromatograms under UV 254 nm (top) and under visible light after derivatization (bottom); track 1, 2, 7 und 8: caffeine standard solutions 50–300 ng/µL; track 3–6: energy drinks; repetition of the analysis on the right half of the plate (track 9–16)

The confirmation resulted from the elution of the sample zones with the TLC-MS Interface into the expression CMS. The mass spectrum showed the protonated molecule at *m/z* 195.12 $[M+H]^+$ and its acetonitrile adduct at *m/z* 236.06 $[M+ACN+H]^+$.



2

HPTLC-MS of a caffeine zone in an energy drink (200 ng/zone)

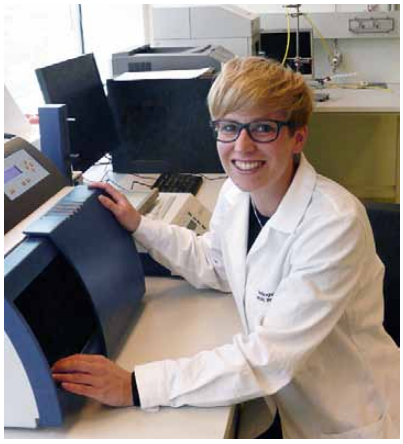
Reduced sample preparation in combination with parallel sample analysis, low cost, short analysis time and substance confirmation allow an effective analysis of caffeine in energy drinks. The simultaneous determination of other ingredients like riboflavin, pyridoxine, nicotine amide and taurine is possible in a single chromatography run [1].

[1] M. Aranda, G. Morlock, J Chromatogr A 1131 (2006) 253

Further information is available from the authors Michael Schulz, Michaela Oberle, Katerina Matheis and Hans Griesinger on request.

Contact: Michael Schulz Merck KGaA, MM-LAR-A, Frankfurter Str. 250, 64293 Darmstadt, Germany, michael.schulz@merck-group.com

HPTLC bioautographic assay for tyrosinase inhibitors in plant extracts



Judith Taibon

Judith Taibon is a PhD student at the Institute of Pharmacy, Department of Pharmacognosy, Center of Chemistry and Biomedicine of the University of Innsbruck, Austria. Within the framework of the EU funded project NATPROTEC she optimized an HPTLC assay for the analysis of tyrosinase inhibitors. The work was done in the CAMAG laboratory in Muttenz, Switzerland, including assay repeatability assessment, stability, reproducibility, and robustness.

Introduction

For the development of innovative cosmeceuticals the research for potentially bioactive plant extracts is of great interest. There are for example plant extracts which inhibit the enzyme tyrosinase in the human skin and thus reduce melanin synthesis. This leads to skin lightening and may remove skin blemishes, like hyperpigmentation and age spots.

For the screening of plant extracts regarding tyrosinase inhibiting activity, an existing HPTLC bioautographic method [1, 2] was optimized and validated. Several method parameters were standardized, including the required amounts of enzyme and substrate, the spraying procedure, and the incubation time.

For the cosmetic industry the effect-directed screening by HPTLC bioautography is an important tool in the search for new bioactive compounds from plants. Compared with the enzyme assay on microtiter plates, the advantages of the HPTLC assay are the separation of the target zone from other substances as well as the plant matrix on the HPTLC plate and its direct accessibility for subsequent analysis like mass spectrometry.

Sample preparation

The plant extracts (obtained by sequential extraction with dichloromethane, ethyl acetate, and methanol, or only with methanol, and evaporated to dryness) were prepared at a concentration of 1 mg/mL in the respective solvent.

Standard solutions

The standards were prepared at a concentration of 1 mg/mL in methanol.

Enzyme solution

For the stock solution with an activity of 12 000 U/mL the required amount of mushroom tyrosinase was dissolved in 1 mL of phosphate buffer. K_2HPO_4-(0.35 %) and $NaH_2PO_4 \cdot H_2O$ (0.28 %)-solutions were mixed 2:3 and the pH adjusted to 6.8. Ten aliquots of 100 µL were made and stored at -20°C. Before use an aliquot was diluted with 2.9 mL of phosphate buffer (activity 400 U/mL).

Substrate solution

0.047 g of L-DOPA were mixed with 20 mL of phosphate buffer containing 1 % Triton X-100 and sonicated for 40 minutes. Stored at 4 °C under light protection the solution can be used for maximum 3 days.

Layer

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

Sample application

Bandwise with the ATS 4, 15 tracks, band length 8 mm, distance from lower edge 8 mm, distance from left and right edge 20 mm. Application volume 2 µL of standards, 15 µL of lipophilic plant extracts (in dichloromethane or ethyl acetate) and 25 µL of polar extracts (in methanol). Of the direct methanol extracts 15 µL instead of 25 µL were applied. Three different amounts of the positive marker kojic acid were applied spotwise on the left and right edge of the plate after development as system suitability test for the assay.

Development

In the ADC2 with chamber saturation (with filter paper) for 20 min, developing distance 70 mm from lower plate edge. Because separation was affected by the activity of the adsorbent, the plate was conditioned prior to development at 33 % relative humidity for 10 min using a saturated solution of magnesium chloride.

Depending on the extraction solvent and the polarity of the compounds one of the following mobile phases was used:

I dichloromethane – methanol – water 70:30:4 for polar extracts (methanol)

II ethyl acetate – methanol – water – formic acid 50:10:7:1 for methanol extracts containing very polar compounds

III toluene – ethyl acetate – formic acid 80:20:2 for lipophilic extracts (dichloromethane or ethyl acetate)

When formic acid is used in the mobile phase, the plate is neutralized after drying and development by placing it for 10 min in a twin-trough chamber saturated with 32% NH_3. Then the plate is dried with cold air for 10 min (mobile phase II) or 20 min (mobile phase III).

Bioautography

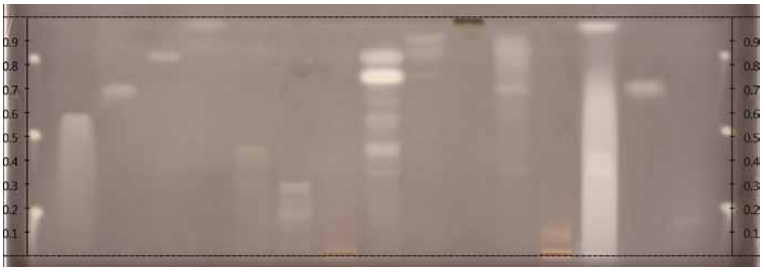
2.5 mL of substrate solution and 3 mL of enzyme solution were successively and homogenously sprayed onto the plate plate sparingly, *i.e.* without creating a liquid film on the plate surface. The sprayed plate was incubated for 10 min at room temperature in an airtight box (to prevent from drying) and then conditioned to <2% relative humidity for 5 min in the ADC 2 using a molecular sieve.

Documentation

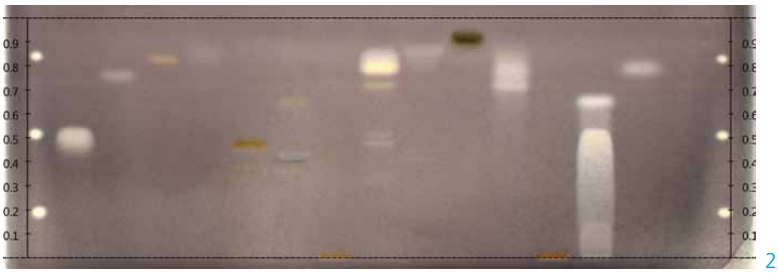
With the Visualizer under UV 254 and 366 nm after development, and under white light (direct mode, transmitted mode, and both combined) after each bioautography step (spraying, incubation, drying).

Results and discussion

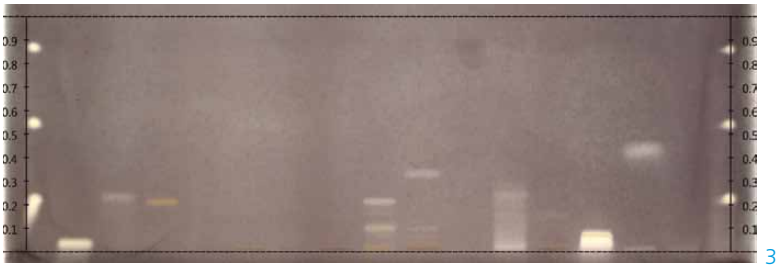
The assay as described in literature was not very practical because the results highly depended on the skill of the analyst. When all parameters were optimized the reproducibility from plate to plate was significantly increased. During the screening of more than 600 extracts, the chromatograms especially of lipophilic extracts revealed many positive results as sharp white zones, which showed a different behavior than known tyrosinase inhibitors. We assumed this was a false positive behavior of poorly wettable lipophilic zones. The problem was solved by adding the non-ionic surfactant Triton X-100 to the substrate solution and by drying the plate after incubation. The chromatograms now showed a homogeneously dark background and their good contrast improved the detectability of the white inhibition zones. Examples of chromatograms developed with each mobile phase are shown in images 1–3. Due to the different mobile phases used the hR_F-values and the intensities of the white inhibition zones are different from plate to plate. In addition the intensity varies due to the different extraction solvents. Some extracts showed intensely colored substance zones which interfered with the detection of tyrosinase inhibition (*e.g.* track 5 on image 2).



Chromatogram developed with mobile phase I, white light (transmission, image electronically enhanced), methanol extracts; track 1: kojic acid, 2: isoferulic acid, 3: resveratrol, 4: paeonol, 5: Euphrasia, 6: Leucanthemum, 7: Cotinus, 8: Morus alba, 9: Glycyrrhiza glabra, 10: Epilobium, 11: Cistus, 12: Pistacia, 13: tropolon, 14: benzoic acid, 15: ascorbic acid



Chromatogram developed with mobile phase II, white light (transmission, image electronically enhanced), methanol extracts; track assignment as above



Chromatogram developed with mobile phase III, white light (transmission, image electronically enhanced), methanol extracts; track assignment as above, except track 10: Chenopodium (instead of Epilobium)

This study was supported by the Commission of the European Community, 7th framework program (PEOPLE - Industry - Academia Partnerships and Pathways), grant agreement no. 286287 NATPROTEC.

[1] Wangthong, S. *et al.*, Biomed Chromatogr 21 (2007) 94

[2] Magnenat C., Development of an HPTLC bioautographic assay for tyrosinase inhibition, Master thesis 2013.

Further Information is available from CAMAG.

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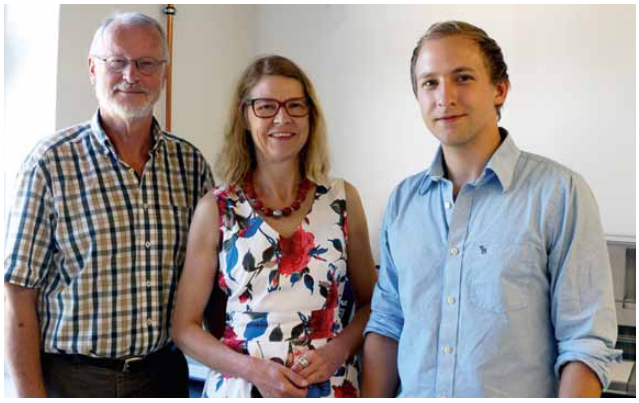
Analysis of plant glycosylceramides by automated multiple development

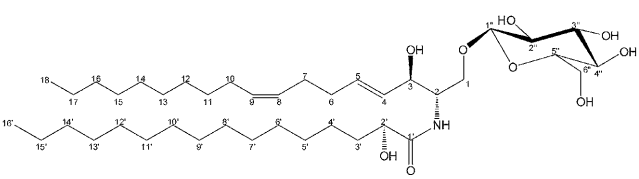


Prof. Dr. Dr. Reinhard Neubert, Prof. Dr. Birgit Dräger, Mathias Reisberg

Professor Dräger and Professor Neubert at the Institute of Pharmacy, Martin-Luther-University Halle-Wittenberg, Germany investigate nature-derived compounds for health improvement and therapy. As a part of his PhD study, Mathias Reisberg investigated the separation and detection of plant glycosylceramides by AMD-HPTLC. In cooperation with the diploma student Peter Chlebowski and the Skinomics Company, he modified an established AMD-HPTLC method for the separation of skin ceramides and transfered it to plant extracts.

Introduction

The outermost layer of the human skin, the *stratum corneum*, serves as a barrier and consists to a high extent of ceramides, a class of lipids. Skin diseases like psoriasis and neurodermatitis appear to arise from disturbed ceramide profiles [1, 2]. Serving as a possible source for therapy, semisynthetic ceramides are very expensive. Inexpensive alternatives might be obtained from plants in the form of glycosylceramides that are additionally glycosylated.



Glycosylceramide Glc-d18:2(4E,8Z) h16:0

Wheat germ, a by-product of wheat milling, was found to be rich in glycosylceramides [3] and used to verify the modified method, based on the 18-step AMD-HPTLC method for skin ceramides by Farwanah *et al*. [4], later improved by Opitz *et al.* [5].

The discovery and (inexpensive) isolation of compounds from nature appear as an attractive way to enhance pharmaceutical therapy options. The AMD-HPTLC method proved to be reliable and effective for screening of plants or quality control.

Sample preparation

Total lipids were extracted from 20 g wheat germs with 100 mL isopronanol – *n*-hexane – water 11:4:5 three times [6]. The first column chromatography on silica gel provided a neutral lipid (chloroform – acetic acid 99:1) and a glycolipid (acetone) fraction. Glycosylceramides were isolated with the third elution of acetone, after discarding fatty acids (chloroform – acetone 4:1) and sterol glycosides (chloroform – acetone 3:2) [3].

Standard solutions

A methanolic lipid mixture (squalene, cholesteryl oleate, glyceryl trioleate, linoleic acid, ß-sitosterol, ß-sitosterol glucoside and glycosylceramide, Glc-d18:2 h16:0) and a ceramide mixture (Cer [AP]-C18, Cer [AS]-C18, Cer [NP]-C18, Cer [NS]-C18, Cer [EOP]-C27 and Cer [EOS]-C27 in chloroform - methanol 1:1) were prepared (100 µg/mL each).

Layer

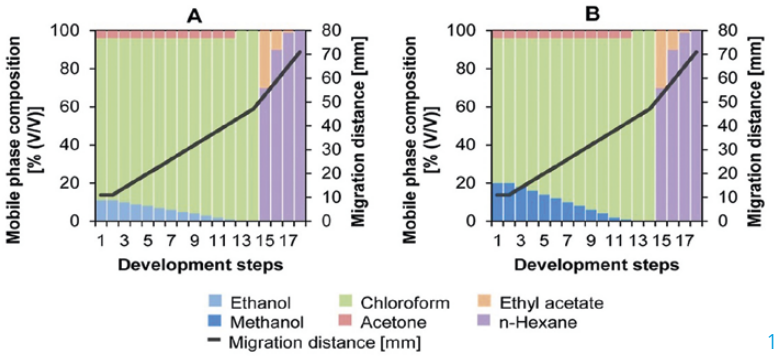
HPTLC plate silica gel 60 F_{254} (Merck), 20 × 10 cm, prewashed by immersion in isopropanol at least for 2 h and dried for 30 min at 130 °C.

Sample application

Samples (10 µL) were applied as 6 mm bands using the Automatic TLC Sampler 4 (ATS 4). The track distance was 10 mm, the distance from lower edge 8 mm and from left side 15 mm.

Chromatography

An 18-step gradient in the Automated Multiple Development 2 (AMD 2) system up to a migration distance of 71 mm [5] was modified. Methanol replaced ethanol, and the mobile phase composition was changed slightly (pre-conditioning with 4 M acetic acid before each step, drying time 1.5 min, development duration 3 h and solvent consumption 201 mL).



1

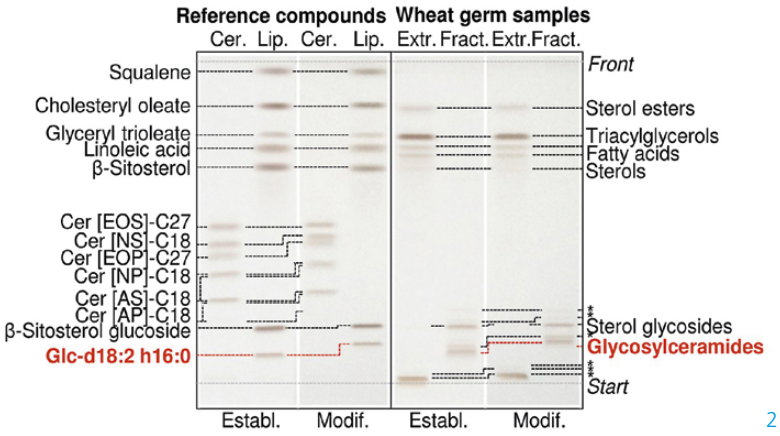
Established [5] (A) and modified AMD-HPTLC method (B)

Post-chromatographic derivatization

Immersion in copper sulfate phosphoric acid reagent for 20 s and heating at 130 °C for 15 min revealed grey-brown bands.

Densitometry

Absorption measurement at 546 nm with TLC Scanner 3 and winCATS, slit dimensions 4 x 0.2 mm, scan speed 20 mm/s



2

AMD-HPTLC chromatograms of standard mixtures and wheat germ samples (ceramide classes in accordance with [1], asterisks represent unknown compound classes)

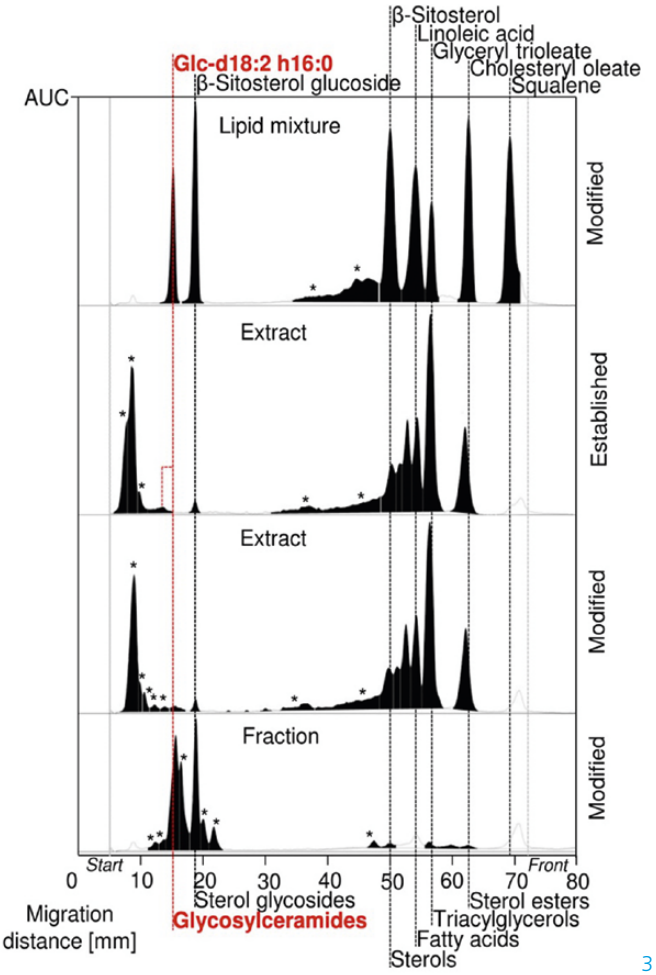
Results and discussion

An AMD-HPTLC method was slightly modified for determination of lipid classes from wheat samples with special regard to the more polar glycosylceramides, whereas the unpolar separation in the upper hR_F range was not changed. Using methanol instead of ethanol led to sharpened bands. The increase of the polar solvent ratio in the first steps accomplished higher hR_F values for the polar compounds and thus achieved a better separation from the potential plant matrix. In the same gradient, plant glycosylceramides were distinguishable from skin ceramides.

For both methods, the acetone fraction showed two distinguished bands of glycosylceramide and sterol glycosides together with some unknown compounds. However in the densitogram of the modified method, small amounts of glycosylceramides could directly be detected in the crude extract samples using the modified method (not visible in the densitogram of the established method). Thus, extensive fractionation could be omitted and the raw lipid extract could directly be used for screening.

For Glc-d18:2 h16:0, regression analysis ($y = -0.009x^2 + 19.434 x + 199.97$) showed a polynomial relationship with coefficients of determination (R^2) from 0.995 to 0.999 (n=3, 50 - 1000 ng/band). LOD (S/N 3) and LOQ (S/N 10) of Glc-d18:2 h16:0 were determined to be 10 and 50 ng/band, respectively (n=6).

The modified method, slightly increased in polarity, could directly detect and quantify the glycosylceramide class in the crude lipid extract and can be used for plant screening and evaluation of the most adequate natural resource for rare and expensive ceramides.



3

AMD-HPTLC densitograms of the lipid mixture and wheat germ extract (established versus modified method) and fraction

[1] Motta *et al.*, Biochim Biophys Acta 1182 (1993) 147

[2] Imokawa *et al.*, J Invest Dermatol 96 (1991) 523

[3] Sullards *et al.*, J Mass Spectrom 35 (2000) 347

[4] Farwanah *et al.*, J Chromatogr B 780 (2002) 443

[5] Opitz *et al.*, Chromatographia 73 (2011) 559

[6] Markham, Jaworski, Rapid Commun Mass Spectrom 21 (2007) 1304

Further information is available from the authors on request.

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4

CAMAG Automatic Developing Chamber ADC 2

The Automatic Developing Chamber offers convenience, safety and reproducibility for the isocratic development of HPTLC plates preferably 20 x 10 cm, with limitations also 10 x 10 cm.

- Due to the chamber geometry and homogenity of the gasphase, identical developing distances from plate to plate are secured and thus reproducible results. Chromatography occurs in a closed system and is therefore independent of environmental conditions.
- The actual developing chamber is identical with a regular CAMAG Twin Trough Chamber, so that analytical procedures can be readily transferred in both directions.
- Preconditioning of the layer, the chamber saturation as well as final drying is fully automatic with pre-set parameters.
- The user is freed from all process monitoring responsibilities, a CCD sensor surveys the solvent migration distance.
- The option "Humidity Control" offers investigation of the Influence of relative humidity during method development.

Further information on **www.camag.com/adc2**

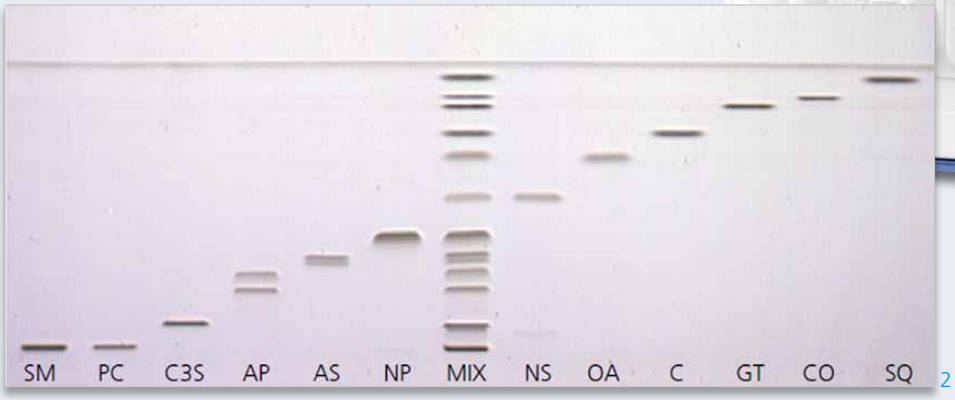
CAMAG AMD 2 System

Automated Multiple Development of planar chromatograms

1

Principle

- The HPTLC plate is developed repeatedly in the same direction.
- Each successive run extends over a longer solvent migration distance than the one before.
- Each successive run uses a solvent of lower elution strength than the one used before.
- Between runs the solvent is removed and the plate is completely dried.



2

The chromatogram shows the strength of the AMD 2 on separating compounds with a wide polarity range (e.g., the separation of ceramides, cholesterol and fatty acids from stratum corneum, CBS 105). This technique results in extremely narrow bands over the whole separation distance, especially visible for compounds showing a high hR_F value. It also enables more components to be baseline separated per track.

Result

- Due to the stepwise elution gradient, combined with the focusing effect of the subsequent runs, extremely narrow bands are formed with typical peak widths of about 1 mm.
- Over the available separation distance of 80 mm up to 40 components can be baseline separated.
- This ensures the highest resolution that can be attained with a planar chromatography system.

In two applications presented in this CBS 113 AMD 2 was employed. One describes an HPTLC/AMD screening method for the identification of impurities of unknown origin in water. The other one shows the separation of plant glycosylceramides and skin ceramides with an 18-step AMD gradient optimized for this purpose.

Further information: **www.camag.com/amd2**

CAMAG WORLD LEADER IN PLANAR CHROMATOGRAPHY

CBS 113 E 996.2270-16