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Planar Chromatography
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Procedures, applications

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Column: Know CAMAG

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Modern direct bioautography of endocrine active compounds

M. Sc. Ines Klingelhöfer and Prof. Dr. Gertrud Morlock, Institute of Nutritional Science and Interdisciplinary Research Center of the Justus Liebig University Giessen, are active members of the Expert Group for the development of the yeast estrogen screening in combination with planar chromatography (HPTLC-pYES). In July 2013, they showed a direct bioautography method (DB) which led for the first time to sharp substance zones. By such a meaningful, effect-directed screening of complex samples and the subsequent identification of these bioactive substances, an extensive food and natural product screening is now feasible.

Introduction

Endocrine active compounds are ubiquitous in our food, in nutritional supplements and in cosmetics. They affect the health of humans and animals by controlling and regulating essential functions of metabolism, growth and development. These substances include both natural estrogens, such as ß-estradiol and estrone, which pass through excreta of humans and animals in the environment and therefore in the food circulation, as well as plasticizers, pesticides and biocides, which contaminate the food through industrial production. Another group of substances are phytoestrogens – phytochemicals in nature. A specific detection method for these substances that act like estrogens is HPTLC-pYES.

The bioautography has been used for almost 70 years [1], however, the strong diffusion of the substance zones (during several hours of incubation with the aqueous culture medium) has been a substantial disadvantage. Any attempt to improve the DB was unsuccessful, and highly diffuse zones were not really convincing to the analyst.

The DB shown here, using the example of HPTLC-pYES, was substantially improved and for the first time carried out on RP-18 W plates [2, 3], which were considered to be not applicable for the DB (as these phases did not show the bioassay response). Since water has no elution strength on the reversed phase, and long incubation times in an aqueous medium result in less diffusion of the zones, we pursued to use water-wettable reversed phases. These as well as medium-polar layers appear clearly more suitable for DB than the previously used silica gel phase. The improved HPTLC-pYES method detects estrogen-effective substances as sharply-bounded

zones in complex sample matrices. The excellence of these new DB methods is illustrated by the fact that the biological detection can also be used for quantification.

Sample preparation

Propolis tinctures were used directly or diluted 1:10 with ethanol. The content of propolis capsules was treated with 1 mL ethanol, mixed for 3 min (vortex), centrifuged at 15,000 g for 5 min and the supernatant analyzed. Propolis lozenges were pestled and 100 mg of this material was extracted analogously to the capsules.

Standard solutions

Estrone (E1), 17-estradiol (E2), 17-ethinylestradiol (EE2), estriol (E3), bisphenol A (BPA), 4-*n*-nonylphenol (NP) and caffeic acid phenethyl ester (CAPE) were dissolved in ethanol or methanol (0.1, 1 and 50 pg/µL and 2.5 and 50 ng/µL).

Chromatogram layer

HPTLC plate silica gel 60 RP-18 W (Merck), 20 x 10 cm, cut when necessary with the smartCut plate cutter

Sample application

Bandwise with Automatic TLC Sampler (ATS 4), band length 6.5 mm, track distance 7.5 mm, distance from lower edge 8 mm and from the lateral edge 12 mm, application volume 0.1 µL/band (standards) and 0.1 – 5 µL/band (samples)

Chromatography

In the Twin Trough Chamber 10 x 10 cm or 20 x 10 cm with 5 mL or 10 mL *n*-hexane – toluene – ethyl acetate 8:3:2, migration distance 7 cm

Bioassay

The chromatogram was immersed into the yeast cell suspension (recombinant *Saccharomyces cerevisiae* BJ3505 cells, which express the human estrogen receptor (hERα) and carry the reporter gene lac-Z) and incubated for 3 h [2]. Estrogen-effective substances result in the release of β-galactosidase, which cleaves the substrate pyranoside (placed by a second immersion step) to 4-methylumbelliferyl-β-D galactopyranoside to the blue fluorescent dye 4-methylumbelliferone (MU).

Biodensitometry

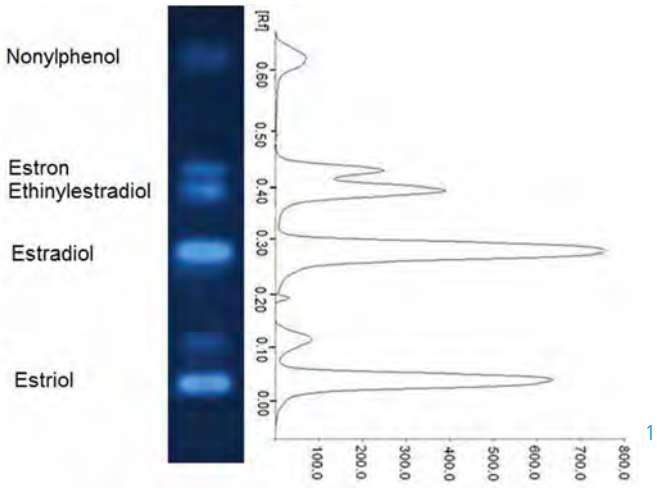
TLC Scanner 3 with winCATS, fluorescence measurement at 365/>400 nm, mercury lamp, slit dimension 5.0 x 0.2 mm, scanning speed 20 mm/s, evaluation via polynomial regression

Mass spectrometry

Elution of the bioactive zones with methanol-ammonium formate buffer (10 mM, pH 4, 49:1, 0.2 mL/min) by TLC-MS Interface (elution head 4 x 2 mm) into the ESI-MS (CMS, Advion)

Results and discussion

For the detection of estrogen-effective substances in matrix-rich samples, up to 24 samples were separated on the HPTLC plate in parallel. The optimization of the HPTLC-pYES workflow and adaption of the yeast cell cultivation for the RP-18 W HPTLC plate was carried out with the standard substances E1, E2, EE2, E3 and NP. This resulted in sharp, blue fluorescent MU-zones.



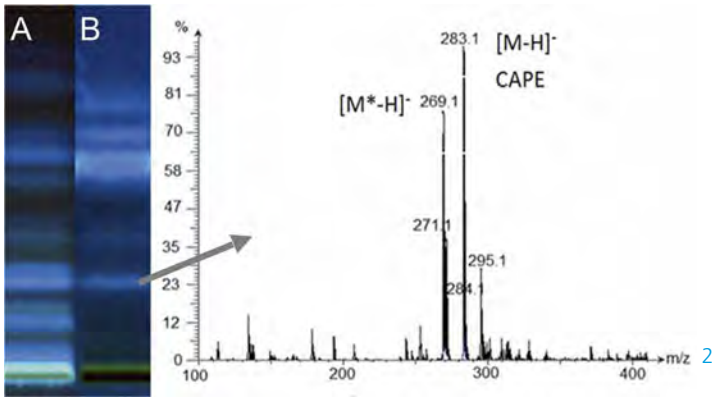
Sharply-bounded, blue fluorescent MU zones after 4 h of incubation in an aqueous medium; fluorescence measurement at UV 365/>400 nm

The response (blue MU-fluorescence) was generated by the activity of the yeast cells and subsequently measured conventionally (biodensitometry). LOD, LOQ and the maximal working range of the estrogen-effective substances were determined ($n = 3$, each with a newly prepared yeast cell culture) [2]. The detection of E2 was the most sensitive: depending on the method of calculating, the average LOD was 0.2 pg/band and the mean LOQ at 0.5 pg/band (calibration method in accordance

with DIN 32645) or at 0.5 and 1 pg/band, if it was calculated over the signal-to-noise ratio (S/N).

Substance (pg/band)						
Mean (n=3)	E1	E2	EE2	E3	BPA	NP
LOD (S/N 3)	25	0.5	2	n.d.	62.5×10^2	25×10^3
LOQ (S/N 10)	50	1	5	500	12.5×10^3	50×10^3
Working range	$25 - 25 \times 10^3$	0.5 – 50	$2 - 1 \times 10^3$	$5 \times 10^2 - 50 \times 10^3$	$62.5 \times 10^2 - 1 \times 10^6$	$25 \times 10^3 - 1 \times 10^6$

Using seven commercially available propolis samples, it was demonstrated that with no or only minimal sample preparation estrogen effective substances could be detected with excellent sensitivity, identified and quantified [3]. Many food extracts, such as the propolis samples shown here, showed natively blue fluorescent zones [2, 3]. For the exclusion of false positive MU-zones, the entire bioassay procedure was carried out without yeast cells. The corresponding negative controls showed no natively fluorescent zones. The implementation of a negative control was considered essential to guard against false positive findings.



2

Chromatograms of a propolis sample: natively fluorescent zones (A) and blue fluorescent MU-zones for HPTLC-pYES (B); exemplary identification of a zone (CAPE) by ESI-MS (TLC-MS Interface)

CAPE was found in all propolis samples. For quantification, the recovery in the range of 10 to 150 ng/band was determined. The average recovery of all the concentration levels was 95 % (*%RSD* = 15 %; $n = 7$). Via biodensitometry (5-point calibration), the CAPE contents of propolis samples corrected over the average recovery rate were between 710 and 2387 µg/g and agreed very well with literature values (using SPE and HPLC-MS/MS) [3]. Through this optimized DB method, it was possible for the first time – due to the sharply defined zones – to detect individual bioactive ingredients in complex samples with sufficient sensitivity to make a unique assignment and to identify and to quantify response generated by the yeast cells. Discovered active ingredients are easier to quantify by conventional detection modes (physically/chemically), if compared to biodensitometry. The latter served here as proof of the achieved performance quality, accuracy and reliability of this effect-directed analytics.

It is good scientific practice, to carry out the LOD and concentration information by biodetection via multiple analyses, comparing the values of each newly prepared yeast cultures. LODs in very good working yeasts were likewise reproducible at 250 fg/band E2 (S/N 3), but were not always reachable by newly prepared yeast cultures.

For important estrogen-effective substances the LODs are in the fg- and pg-range and thus allow the direct detection at trace levels without enrichment.

Further information is available on request from the authors.

[1] R.R. Goodall, A.A. Levi, Nature 158 (1946) 675

[2] I. Klingelhöfer, G.E. Morlock, J Chromatogr A 1360 (2014) 288

[3] G.E. Morlock, I. Klingelhöfer, Anal Chem 86 (2014) 8289

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Quantification of xanthones in mangosteen fruit hull extracts

From left: Aline Gilbert, Dr. Benedicte Portet, Jocelyn Giboulot

All research activities for the Rocher group are concentrated at the new Yves Rocher Research Center. The aim of this center is to develop new cosmetic products from the group's brands. The laboratory of phytochemistry is integrated into the botanical innovation department whose future goal is to study all aspects of active plant extracts and active ingredients. For some years this laboratory has advocated HPTLC and been in close connection with the French HPTLC Club, hosting its last meeting. They were also granted the First Prize for poster presentation during the HPTLC Symposium in Lyon, July 2014.

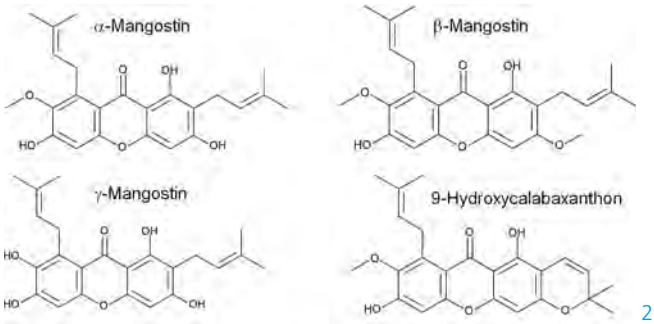
Introduction

Mangosteen (*Garcinia mangostana* L., *Clusiaceae*) is the so-called "queen of tropical fruits" due to its remarkably pleasant flavour and is native to Malaysia and Indonesia. Previous experimental studies have demonstrated that extracts of the brownish-purple fruit hull have antioxidant, anti-inflammatory and antibacterial properties. Phytochemical investigations attribute these pharmacological activities to its xanthone analogues, the main bioactive secondary metabolites of mangosteen [1]. The hull contains more than 60 xanthones and the main compounds are: α-mangostin, γ-mangostin, β-mangostin and 9-hydroxycalabaxanthone.

The aim of this study was the characterization and separation of the four main xanthones in crude ethanolic extracts of mangosteen hulls by HPTLC, followed by quantification with densitometry. At the same time a quantitative HPLC method was developed. We obtained reliable results with both techniques, but the HPTLC method was easier and faster for the testing of raw materials.

1

Mangosteen, the fruit of Garcinia mangostana L.



2

Main xanthones of mangosteen fruit hull

Chromatogram layer

HPTLC plates silica gel 60 RP-18, 20 x 10 cm

Standard solutions

Xanthones were dissolved in ethanol (α-mangostin 0.06 mg/mL and γ-mangostin 0.05 mg/mL).

Sample preparation

Dried fruit hulls from India were grinded to a particle size between 4 and 0.5 mm. Ground hulls were extracted in ethanol (plant/solvent ratio 5:95, *w/w*) for 2 h in reflux and filtered through a cellulose membrane (5–10 µm). After solvent evaporation, the dry extracts were stored in the dark at room temperature. For analysis, the extracts were dis-

solved in ethanol (4 mg/mL). For HPLC analysis, extracts (92.5 mg/mL in methanol) were filtered through a PTFE membrane (0.45 µm).

Sample application

Bandwise with Automatic TLC Sampler (ATS 4), band length 7 mm. For calibration, between 5–15 µL of α-mangostin and between 15–25 µL γ-mangostin were applied.

Chromatography

In the Automatic Developing Chamber (ADC 2) with acetonitrile – water – formic acid 80:17:3, migration distance of 65 mm.

Post-chromatographic derivatization

After drying, the plate was sprayed with NEU´s derivatization reagent and PEG.

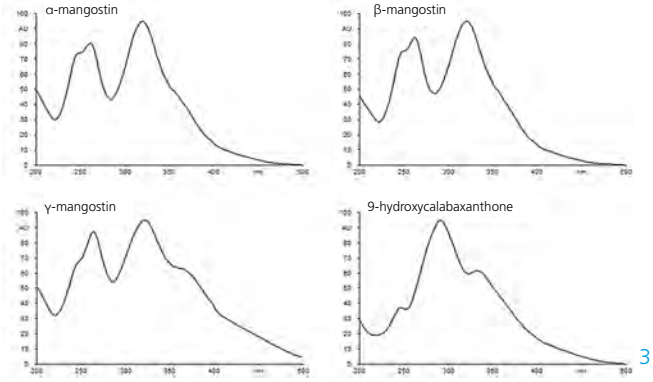
Editor's note: For quantification, a homogeneous application of the derivatization reagent is recommended. Automated immersion is better suited than manual spraying.

Documentation

With the Digistore 2 under UV 366 nm

Densitometry

TLC scanner 3 and winCATS, absorption measurement at 280 and 320 nm. The total amount of the four xanthones was calculated as α-mangostin equivalents (peak area).



3

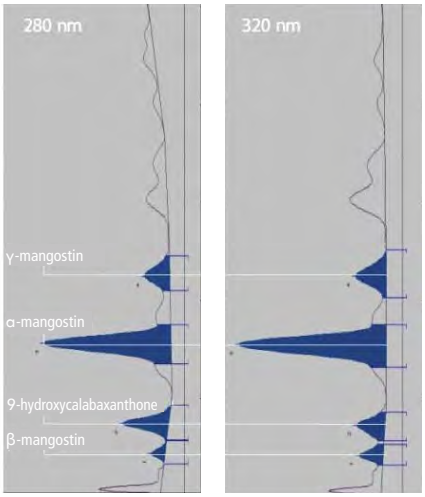
UV/Vis spectra of the four xanthones recorded

HPLC-UV

Using an Alliance model 2695 (Waters), separations were achieved on a LiChrospher RP-18 column (250 x 4.6 mm, 5 µm, Merck) using a gradient of 80 % → 0 % acetonitrile and 20 % → 100 % water, containing 0.1 % *o*-phosphoric acid (85 %) and detection at UV 280 nm (2996 photodiode array detector). Quantification was carried out via calibration curves of γ-mangostin from 0.062 to 0.01 mg/mL and 0.185 to 0.009 mg/mL for α-mangostin. Sample extracts of 20 µL were injected and analyzed in duplicate.

Results and discussion

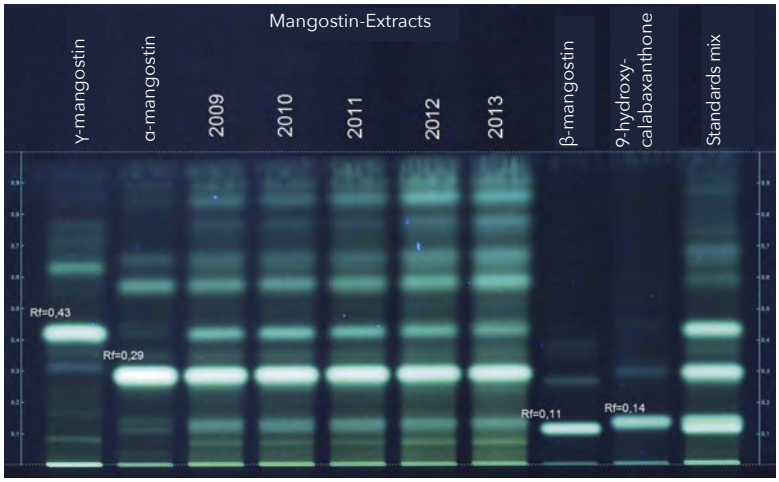
The analysis of samples of five different harvest years (2009–2013) revealed that the extraction yield was mostly around 10 %. Due to the polarity of the differently prenylated xanthones, the best separation was obtained on HPTLC plates RP-18. The UV/Vis spectra of α-mangostin, γ-mangostin and β-mangostin showed three maximal absorption bands at 245, 261 and 320 nm, while 9-hydroxycalabaxanthone showed those at 291 nm and minor at 245 and 332 nm. As a compromise, measurement was performed at 280 and 320 nm. Although signals obtained at 320 nm were slightly higher than those obtained at 280 nm, no significant difference in the result was found.



4

HPTLC densitograms of a mangosteen hull extract at 280 and 320 nm

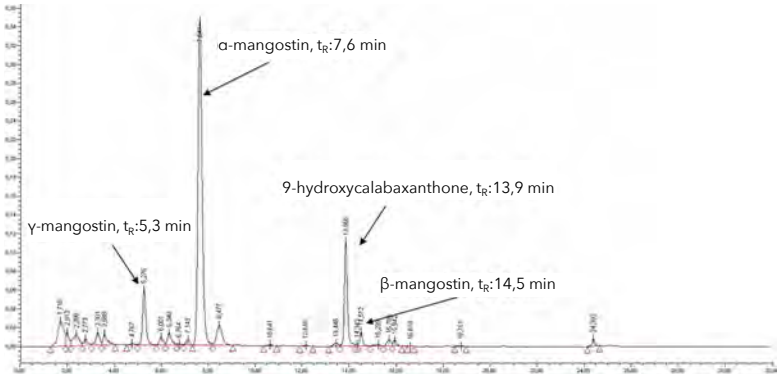
With regard to quantification, the 1:11 (for γ-mangostin) and 1:16 (for α-mangostin) wide concentration ranges allowed flexibility with regard to sample application volumes. α-mangostin (between 32 and 43 %) and γ-mangostin (between 4 and 6 %) were the main xanthones in the samples, as reported elsewhere [1, 2]. The samples originated from the same harvest area, and thus, the variance in the xanthone content was explained by the different climates of the five years and the natural variability of the plant.



5

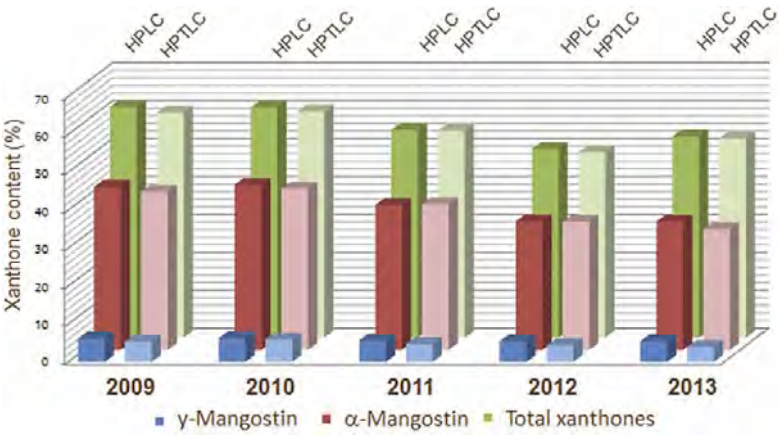
HPTLC RP-18 chromatogram at UV 366 nm after derivatization with NEU's reagent

Phytochemical analyses were also performed by HPLC-UV. The comparison of the phytochemical profile at 280 nm showed that the elution and the separation of the targeted compounds were quite similar for the two analytical methods.



6

HPLC chromatogram of a mangosteen hull extract at 280 nm



7

Xanthones' content in ethanolic hull extracts analyzed via HPLC and HPTLC

With regard to quantification, the HPLC results were higher (+2 % for α-mangostin, +16 % for γ-mangostin). However, the total content differed only by a mean value of 1.5 % between HPLC and HPTLC. Hence, HPTLC was selected as a good alternative to HPLC for the quantification of the four xanthones in mangosteen hull extracts. The HPTLC method developed was rapid and easy to perform. It was considered as a powerful analytical tool for quality control of incoming mangosteen hulls, used as raw material for dietary supplements and cosmetic applications.

Further information is available on request from the authors.

[1] O. Dmitriy *et al.* Phytother Res 23 (2009) 1047

[2] J. Pedraza-Chaverri *et al.* Food Chem Toxicol 46 (2008) 3227

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Dr. Markus Wyss is the new Chief Executive Officer

Dr. Markus Wyss joined CAMAG in January 2013 in the function of Head Sales & Marketing, as has been reported in CBS 111. As foreseen and planned at that time, the Board has appointed him Chief Executive Officer effective 1. July 2015. He will continue his function as Head Sales & Marketing.

CAMAG Research & Development under new management

Nicolas Richerdt
Head Research & Development

Nicolas Richerdt joined the CAMAG software team in early 2008. Very rapidly he became thoroughly familiar with our new generation software *visionCATS* and assumed the duties of it's architect. When the Head of Research & Development position became available, we decided to give him the chance to prove his competence in this strategic function. We wish for him good luck and much success! It is reassuring to know that all members of his team stand behind him and value his total commitment. His strategic thinking and future oriented actions have brought him wide spread acceptance. We are convinced that his activities as the Head of Research & Development will give CAMAG dynamic new life.

Dr. Konstantinos Natsias
Chairman of the Board

Nicolas Richerdt completed his bi-national studies of software development, automation and electronics in Mulhouse/France and Montreal/Canada in 2001 as Master of Science. Parallel to his industrial occupation he completed his MBA degree in 2013 at the Edinburgh Business School (Heriot Watt University). After his studies he acquired wide spread industrial experience in the field of automation and IT business. This background provides optimal experience for the challenging management tasks that await him at CAMAG.

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While compilation of this issue, a variety of applications have been reported from pharmaceutical analysis to archaeological investigation and phytochemical analysis. Also the determination of the total cholesterol in both healthy and cancerous human lung tissue was performed by HPTLC. An interphase model for retention widens the view on fundamentals and provides insight into the relative importance of various intermolecular interactions on retention and selectivity. So, for normal phase separations, contributions of solvent and solute interactions with the adsorbent surface have to be considered.

As more and more validation data are reported for the methods used, reflection on the significance of digits is important for a proper report and interpretation of the results. Of course, the number of decimal figures is dependent on either the calculated measurement uncertainty or the precision of the results for repeated analyses. Thus, reproducibility rates as well as LOD and LOQ values can be rounded to the integer.

Another issue refers to nomenclature, when publishing. According to IUPAC, the R_F value – or preferably the hR_Fvalue – has to be written in italics with a subscript, non-italics F. For the reporting of abstracts in the CCBS database, we tolerate nonconformity to IUPAC writing with regard to the otherwise immense effort.

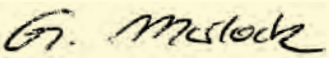
We are frequently asked how to receive the CBS hardcopy. Please feel free to write a short note, including your delivery address, to cbs@camag.com and you will get the current CBS as published twice a year.

Dear friends

Half a century has passed, since our company founder Dr. Dieter Jänchen decided to bring out a periodical directed at the advancement and transfer of sound, relevant information about the technique and the application of planar chromatography. The first edition of CBS, May 1965, consisted of four pages and referenced 48 papers. CBS is now and for some time published twice a year. It is read worldwide and has become, due to its information content, much more than a company magazine. The collection of abstracts on important TLC/HPTLC work (CCBS) has evolved into an extensive database with international appreciation. Each new issue presents the latest applications in different fields that are interesting to our readers. Dr. Jänchen was responsible as editor for all issues up to CBS 84 (March 2000). He sought topicality, yet relevance and a high standard of quality for each issue. We thank Dr. Jänchen for the wise initiation of the CAMAG Bibliography Service 50 years ago and for his support for each CBS issue up to the present day.

The current CBS issue presents five up-to-date HPTLC applications in practice, starting with the Yeast Estrogen Screen in combination with planar chromatography (HPTLC-pYES), the characterization and separation of the main xanthons from raw extracts, the quality control of traditional Chinese medicine by image evaluation, the in-process control for production of new ergolin psychedelics via HPTLC-MS and characterization of marker compounds in Java tea.

Kind regards



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   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**
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    c) Water pollution
    d) Soil pollution
38. **Chiral separations**

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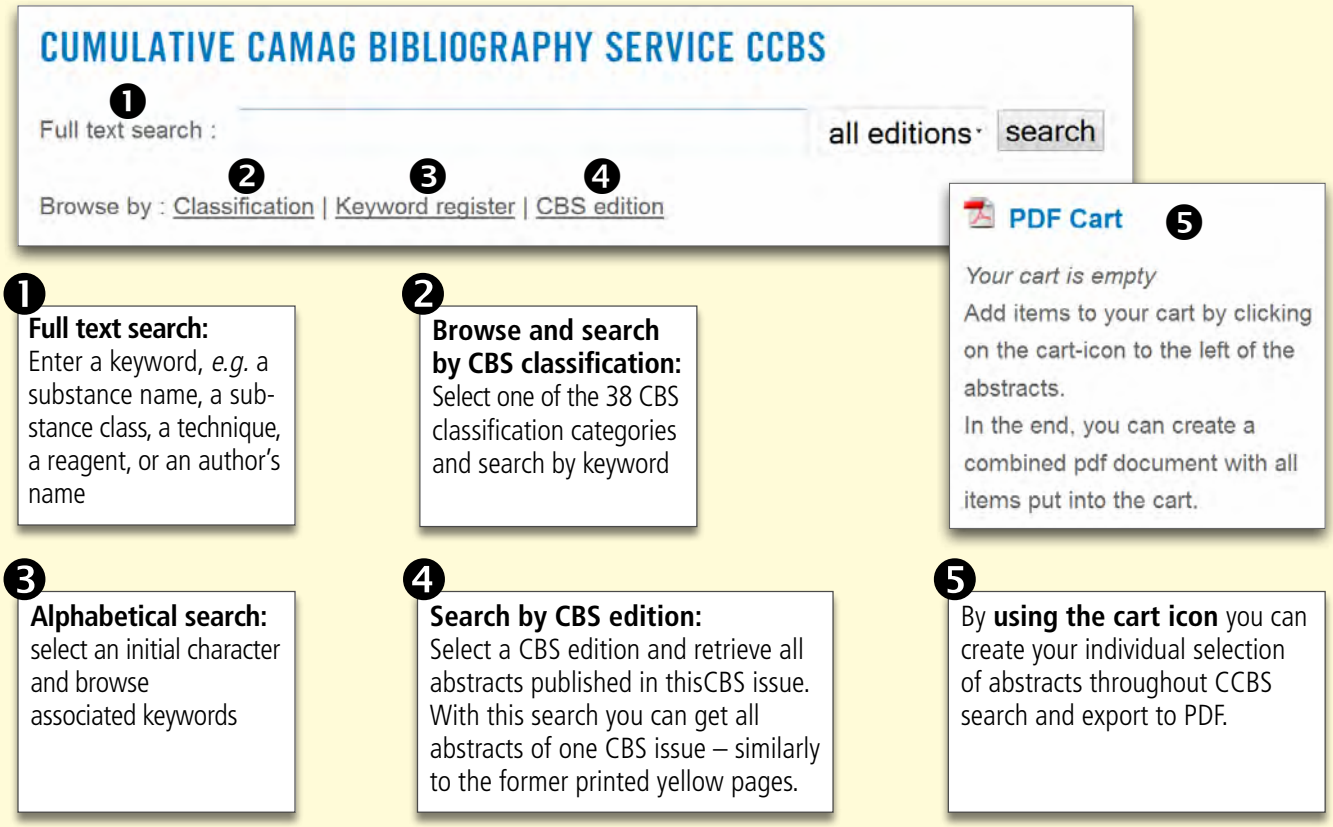

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The database covers most relevant scientific journals in the field of Planar Chromatography including also various non-English publications in German, French, Spanish, Portuguese and Chinese. The CCBS features additional practical information for the 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.

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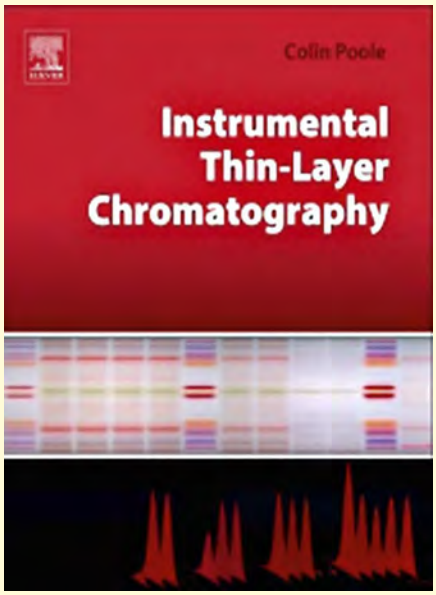
Recommended books



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Instrumental Thin-Layer Chromatography

Colin Poole

Instrumental Thin-Layer Chromatography covers the fundamental principles of TLC and the latest developments and ways to use this method. The book is divided into 22 chapters. Numerous published articles are summarized, with each chapter devoted to a separate article. This gives the reader excellent insights into the different topics. All of the chapters contribute to strengthening our understanding of TLC and its possibilities. The presentation of higher degrees of automation and the linking of these with various detection methods, such as mass spectrometry, encourages the use of innovative approaches for tackling an array of issues.

The individual chapters are superbly organized and the theoretical principles of the different subjects are explained in detail and, for the most part, supported by mathematical formulas and graphs. Chapters dealing with methods development and validation, as well as extensive charts on stationary and mobile phases, derivatization techniques and a detailed description of various development and detection methods with different levels of automation, support the reader and the user in developing a specific method for a specific topic. Chapters 14 to 22 provide an overview of sample applications with numerous up-to-date literature references.

Comparisons with HPLC enable the pros and cons of both methods to be weighed against one another. At times the comparisons are somewhat one-sided and not always captured in a nutshell. This is understandable as the topic of the book is TLC. The book is surely not an easy read for TLC novices since the wealth of information and the details can be overwhelming. On the other hand, the book is perfectly suited to scientists with experience in chromatography for whom TLC represents an important tool in a broad repertoire of analytical methods.

Altogether it can be said that because each chapter focuses on a different area of application, important information and literature references are summarized effectively. The many figures and charts skillfully illustrate the information. On the whole it is a very good book which is recommended to every analytical lab for use as an important source of reference.

Prof. Dr. Ingo Schellenberg

Anhalt University of Applied Siences
Bernburg, Germany

The unique merits of HPTLC image analysis for quality control of herbal medicines

Prof. Xie

Prof. Pei-Shan Xie (Xie Peishan) is renowned worldwide as one of the early pioneers of HPTLC. More than thirty years ago, as the scientific director of the Guangzhou Drug Control Institute (Guangzhou, PR China) he was visionary in realizing that its benefits make HPTLC the method of choice for the description and control of the quality of Traditional Chinese Medicines. From that time he worked determinedly for the introduction of HPTLC into the Chinese Pharmacopoeia (ChP). He chaired the creation of the HPTLC Atlas for Crude Herbal Drugs as a complementary publication of the 2010 ChP edition, and he devoted his scientific expertise and research to the development and refinement of HPTLC as a concept and methodology. Since his retirement he has continued to carry out his own research and publishes most stimulating papers. One of those "Value the Unique Merit of HPTLC Image Analysis and Extending its Performance by Digitalization for Herbal Medicines Quality Control" [1] is summarized below.

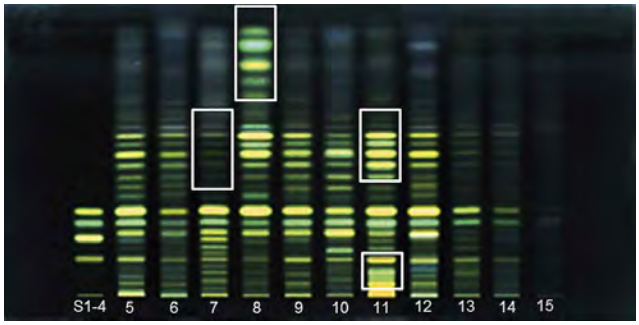
Introduction

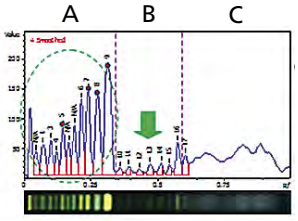
Since herbal drugs and medicines are immensely complex mixtures, their chemical composition is best characterized by so-called chemical fingerprints, which focus on the substances that are known or assumed to have bioactive properties. Chemical fingerprints can be generated by different chromatographic techniques whereby HPTLC is the most flexible and inexpensive of them. Due to its nature as an open chromatographic system that is operated in offline mode, many parameters can affect the outcome of the HPTLC analysis. Therefore, it seems logical that only with a fully standardized operating procedure in place, can the full potential of HPTLC be unlocked. Thus the electronic image of an HPTLC fingerprint, the principal results of analysis, can be evaluated further to explore additional aspects of the quality of herbal medicines.

Example: Differentiation of *Bupleurum* species used as *Bupleurum radix* (Chai hu) by fingerprints and profiles

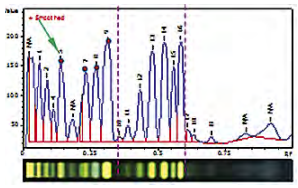
Several species of *Bupleurum* can be used in TCM as *Bupleurum radix* (Chai hu). In addition there are multiple species of the same genus that are not permitted. From a regulatory and safety point of view it is important to distinguish all permitted species from not permitted ones. The major hydrophilic bioactive components of Chai-Hu roots are saikosaponins. Post-chromatographic derivatization with dimethylaminobenzaldehyde reagent induces in these substances fluorescence under UV 366 nm. HPTLC offers an elegant approach to identification and discrimination of all relevant species. Visually their fingerprints can be clearly distinguished. It is possible to divide them into three regions. But it is the conversion of the images into profiles, allowing also the quantitative assessment of the separated compounds that makes discrimination very clear. Region (A) includes 9 peaks of the main saponins. It is shared by all investigated species except *B. longiradiatum*, which contains two more zones. Region (B) can be used to differentiate the species. *B. marginatum var. stenophyllum* has the largest peaks here and *B. longiradiatum* the lowest. Region (C) contains the low-polar constituents. Only *B. bicaule* exhibits six specifically strong peaks in this region.

While proper identification of whole roots of the different species is a task for experts and discrimination of powdered drugs by microscopy is a great challenge, HPTLC fingerprints prove to be a simple yet very powerful tool for species differentiation.

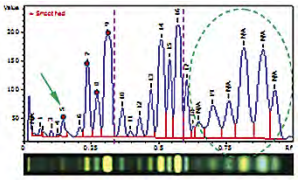




B. longiratiatum



B. marginatum var. stenophyllum



B. bicaule

1

HPTLC of Chai hu , UV 366 nm (track S1–4): saikosaponins f, b2, a and d, (5): Bupleurum chinese, (6): B. scorzonerifolium, (4) B. falcatum, (7) B. longiradiatum, (8) B. bicaule, (9) B. polyclonum, (10) B. wenchuanese, (11) B. marginatum var. stenophyllum, (12) B. falcatum, (13) B. yinchowense, (14) B. simithii var. parvifolium, (15) B. tenue.

For further information, particularly experimental details, please refer to the original publication, which shows three more examples: *Forthysiae fructus, Rehmanniae radix, Salvia miltiorrhizae radix* [1]. The summarized example illustrates the great potential that HPTLC offers for quality control of herbal medicines. The publication of Prof. Xie gives the direction to the future of HPTLC image analysis.

Further information is available from the author.

[1] Xie *et al.* J Chromat Separation Techniq 5 (2014) 249

Contact: Prof. Dr. Pei-Shan Xie, Guangdong UNION Biochemical, Development Ltd. Co. Guangzhou, China, Tel: 86-13352836441, psxie163@163.com

2

visionCATS – Our new generation HPTLC software

visionCATS stands for ease of use and intuitive simplicity. The software organizes the workflow of the HPTLC analysis, controls the CAMAG instruments, and manages data evaluation. Together with CAMAG's HPTLC instruments, *visionCATS* supports a fully standardized operating procedure to achieve reproducible and reliable results – plate to plate, day to day, and lab to lab. The recently published USP general chapters "203" and "1064" define and explain parameters that standardized HPTLC. *visionCATS* provides an HPTLC Method Library with methods that are in full compliance with chapter "203". In addition to these USP methods, others published by the European Pharmacopoeia and by the International Association for the Advancement of HPTLC are included. Other methods will be added continually.

In-process control during synthesis of novel ergoline psychedelics by HPTLC


Dr. Matthias Grill (Lipomed AG), Dr. Melanie Broszat (CAMAG)

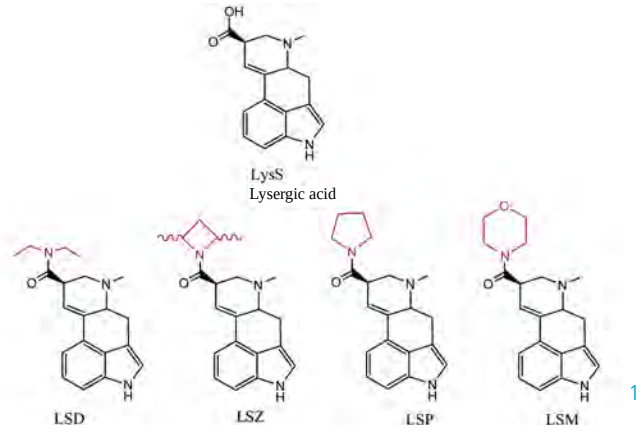
Lipomed AG is a medium-sized company located in Arlesheim, Switzerland. Lipomed produces reference substances and pharmaceuticals for customers worldwide. Dr. Matthias Grill is specialized in the synthesis and purification of forensic substances. In collaboration with CAMAG the benefits of HPTLC for this type of in-process control were determined.

Introduction

TLC has proven to be a convenient tool in classical organic synthesis, though standardized HPTLC offers significantly higher resolution. In combination with hyphenated HPTLC-MS the respective product zones can be reliably confirmed. The aim was to implement HPTLC as a complementary technique to the existing HPLC methods for quality control to ensure a product of high purity.

Product purity cannot be assured with HPLC alone in all cases, due to the fact that side products which do not elute from the column will not be detected. Normal-phase HPTLC is a complementary technique and therefore an established supplement.

Ergot alkaloids are still pharmacologically quite important and represent a highly potent drug class. Dihydroergotamine and cabergoline (remedies for migraine and Morbus Parkinson) are just two examples of these potent agents that carry the ergoline as well as the tryptamine pharmacophore. Since the discovery of lysergic acid diethylamide (LSD) in 1943 by Albert Hoffmann, the psychoactive properties of this active class has been known. Since then a vast number of lysergic acid amides have been synthesized [1,2]. As the existing forensic portfolio has expanded, the most potent analogs of this class have been manufactured. Starting from ergotamine, lysergic acid (LysS) has been extracted and purified. Lysergic acid itself is the starting material for the synthesis of all psychoactive lysergic acid amides. The modification of different amide-side chains results in hallucinogens with a potency comparable to that of LSD.



1

Chromatogram layer

HPTLC plates silica gel 60 F_{254} (Merck), 20 × 10 cm, prewashed by development with isopropanol up to 80 mm

Standard solutions

Methanolic solution of reference substances (0.1 mg/mL)

Sample preparation

Samples were applied directly onto HPTLC plates without sample preparation. Depending on the process step the applied volume varied as well as the solvent (in most cases dichloromethane and/or methanol).

Sample application

Bandwise with Automatic TLC Sampler (ATS 4), 15 tracks, band length 8 mm, track distance 11.4 mm, distance from left egde 20 mm, distance from lower edge 8 mm, application volume between 0.5 und 15 µL for sample solutions and 4 µL for standard solutions.

Chromatography

In the ADC 2 with chamber saturation (with filter paper) for 20 min and after plate conditioning at 33 % relative humidity for 10 min using a saturated solution of magnesium chloride, development with dichloromethane – methanol – triethylamine 9:1:0.002 or in the case of the LSZ with dichloromethane – isopropanol – triethylamine 9:1:0.002 up to the migration distance of 70 mm (from lower plate edge), drying for 5 min.

Documentation

With the TLC Visualizer under UV 254 nm and UV 366 nm

Densitometry

With TLC Scanner 4 and *visionCATS*, absorbance measurement at 254 nm, slit dimensions 5.00 x 0.20 mm, measurement speed 20 mm/s, spectra recording 200 to 500 nm

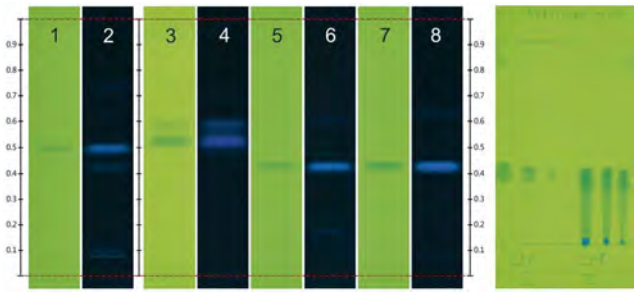
Mass spectrometry

Target zones were directly eluted with the TLC-MS Interface 2 at a flow rate of 0.2 mL/min with methanol (with 0.1 % formic acid) to an ESI-MS (expression CMS, Advion) and detected in the positive ionization mode.

Results and discussion

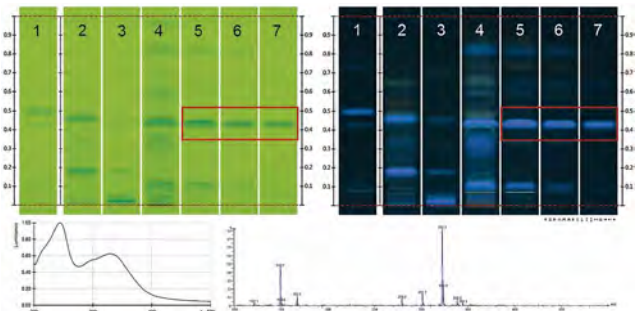
The lysergic acid amides LSZ, LSM and LSP shown above have been synthesized and isolated as tartrate salts in high purity (>99 %). However, as a consequence of multiple purification steps as well as the generic sensitivity of the ergoline structure towards oxygen, light and humidity, the high concentration in the beginning (crude reaction mixture) decreased to approx. 20 %. According to the chromatograms the improved purity grade during the purification workup can be easily observed in the example of the LSP. Furthermore, in the case of LSZ the formation of literature-known isomers (orientation of methyl groups on the azetidine-ring) [1] can be visualized and confirmed by HPTLC-MS. In comparison with the manual TLC technique, the considerably enhanced resolution of the HPTLC under optimized and standardized conditions can be used for illustrating the purity of these sensitive molecules.

During process development for a new synthesis product, valuable information about the individual process steps can be obtained by HPTLC. Visual evaluation of the chromatograms enables a rapid observation on the formation or up- /downscaling of the different components during each step.



2

HPTLC Image Comparison of the purified synthesis products under UV 254 and UV 366 nm (left; tracks 1 and 2: LSD as reference; 3 and 4: purified LSZ (two product zones due to the formation of the isomers [1]), 5 and 6: purified LSM, 7, 8: purified LSP) versus control with TLC (right)



3

In-process control of the LSP: Chromatograms under UV 254 nm and 366 nm show the sequence of the synthesis and the purification steps (track 1: LSD as reference, 2: digest of the lysergic acid from ergotamine, 3: purified lysergic acid (starting material for the chemical synthesis), 4: crude synthesis product (LSP and side products), 5, 6: column purification steps, 7: finished product as well as the UV spectra and the mass spectra of the eluted LSP zone at m/z 322.3 $[M+Na]^+$

Lipomed AG has implemented HPTLC not only for confirmation but also to guarantee high product purity to their customers. This collaborative work has proven HPTLC to be an excellent tool for the establishment and verification of new synthesis processes during this collaborative work.

Further information is available from the authors.

[1] David E. Nichols *et al.*, J Med Chem 45 (2002) 4344

[2] Alexander Shulgin *et al.*, TIHKAL: The Continuation, 1997

Contact: Dr. Matthias Grill, Lipomed AG, Fabrikmattenweg 4, 4144 Arlesheim, Switzerland, Matthias.grill@lipomed.com

Dr. Melanie Broszat, CAMAG, Sonnenmattstr.11, 4132 Muttenz, Switzerland, melanie.broszat@camag.com

Marker compounds in Java tea characterized by HPTLC

René De Vaumas, Extrasynthese and Tiên Do, CAMAG

The subject of this article brings together two companies who have a common interest in sharing the importance of knowledge about excellent phytochemical reference materials, medicinal plants and their constituents, and the use of HPTLC as a standardized analytical method. Extrasynthese is an independent French company with a catalogue of hundreds of reference materials which can be used for regulatory and quality testing, analyzed predominantly with HPTLC. The activities of the CAMAG laboratory in the analysis of phytochemicals are well documented in its dedication to the world-wide recognition and acceptance of HPTLC as the standard method for plant analysis. In this article the focus is on specific markers in method development for the analysis of complex plant extracts.

Introduction

Orthosiphon is an Indonesian medicinal plant which is widely used as an herbal tea commonly known as Java tea. *Orthosiphon* contains high levels of phenolic compounds such as sinensetin and rosmarinic acid. Sinensetin is therefore selected as a reference substance in the monograph of the European Pharmacopoeia, but it can be found in many other herbs as well.

Through investigation of HPTLC fingerprints of flavonoids from *Orthosiphon* species, besides sinensetin, other 5, 6-dimethoxy flavones, and 5-hydroxy-6-methoxy flavones could be identified, which could be used as specific markers for the identification.

Chromatogram layer

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

Standard solutions

Methanolic solutions (1 mg/mL) of sinensetin and eight other reference substances (Extrasynthese); the selection of substances was made on the basis of Extrasynthese's research on flavonoid components and publications [1–3].

Sample preparation

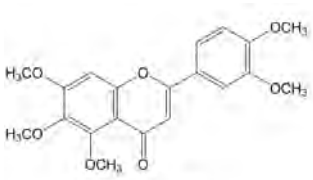
0.5 g of each powdered drug was mixed with 5 mL of methanol and sonicated for 10 min. After centrifugation the supernatant was used as test solution.

Sample application

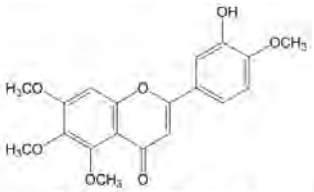
Bandwise with Automatic TLC Sampler (ATS 4), 15 tracks, band length 8 mm, track distance 11.4 mm, distance from left edge 20 mm, distance from lower edge 8 mm.

Chromatography

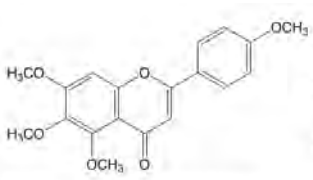
In the ADC 2 with chamber saturation (with filter paper) for 20 min and after conditioning at 33% relative humidity for 10 min using a saturated solution of magnesium chloride, development with toluene – ethyl acetate – methanol 11:8:1 to the migration distance of 70 mm (from the lower edge), drying for 5 min.



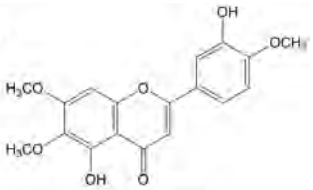
Sinensetin



Eupatorin-5-methylether



Scutellarein tetramethylether



Eupatorin

1

Documentation

With the TLC Visualizer under UV 254 nm and UV 366 nm

Densitometry

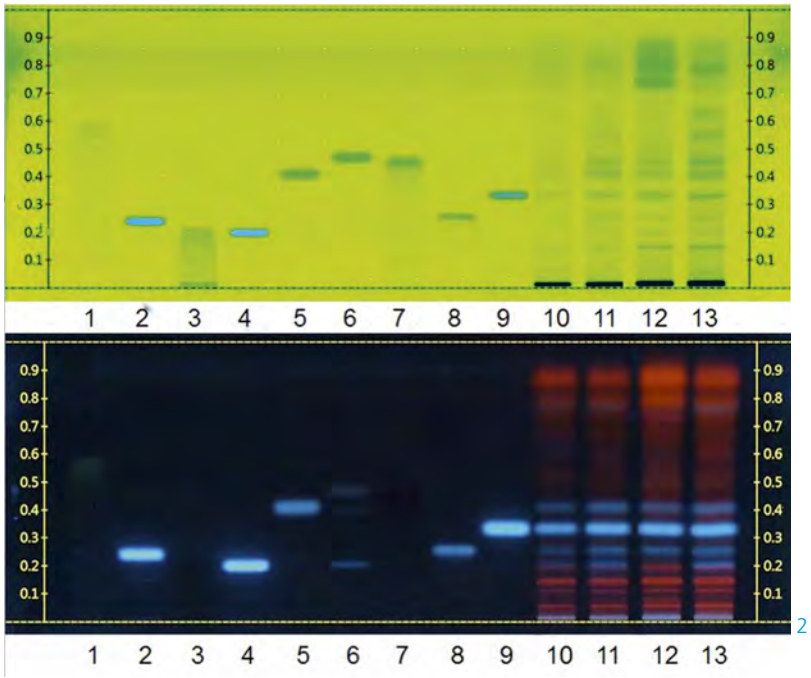
TLC Scanner 4 and *visionCATS*, absorption measurement at 254 nm, slit dimension 5 mm × 0.30 mm, measurement speed 20 mm/s, spectra recording from 190 to 600 nm

Mass spectrometry

Elution of zones with TLC-MS Interface (oval head 4 × 2 mm) into a single mass spectrometer (expression CMS, Advion, Ithaca, NY). Data processing and evaluation of mass spectra was performed with *Advion Mass Express 2.0* and *Advion Data Express* 2.0.50.9

Results and discussion

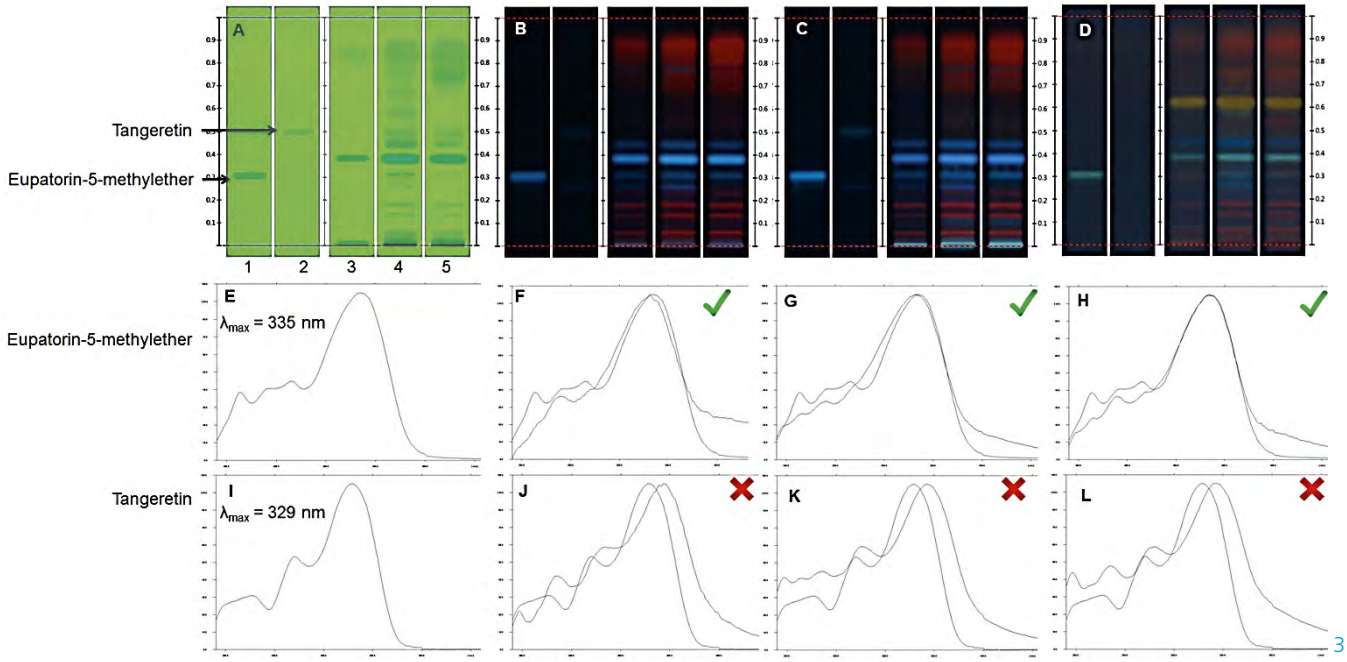
The mobile phase of the European Pharmacopoeia Monograph No. 1229 was used for the separation of 9 available standards (rhamnazin, apigenin-4,5,7-trimethylether, 6-methoxyluteolin, luteolin tetramethylether, scutellarein tetramethylether, tangeretin, eupatorin, eupatorin-5-methylether, sinensetin). Only rhamnazin and 6-methoxyluteolin have shown a tailing.



Documentation under UV 254 nm and UV 366 nm; track 1: rhamnazin; 2: apigenin-4,5,7-trimethylether; 3: 6-methoxyluteolin; 4: luteolin tetramethylether; 5: scutellarein tetramethylether; 6: tangeretin; 7: eupatorin; 8: eupatorin-5-methylether; 9: sinensetin; 10–13: Orthosiphon aristatus

In order to confirm the presence of the selected substances in the samples, different detection modes were tested and the UV spectra recorded. For example, following development with toluene – ethyl acetate – methanol 11:8:1, eupatorin-5-methylether and tangeretin were detected under UV 254 nm and 366 nm prior derivatization, and under UV 366 nm after a two-step derivatization (natural product reagent, then anisaldehyde-sulfuric acid reagent).

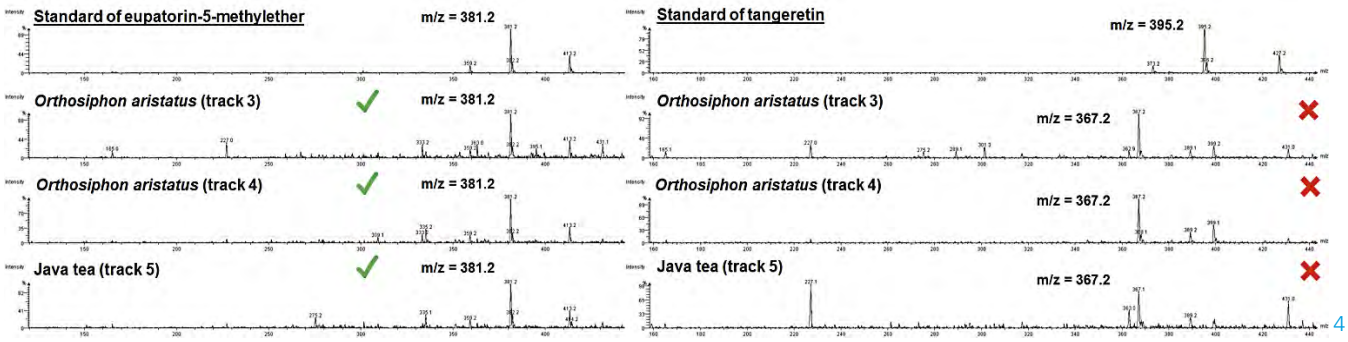
Prior and after derivatization (A–D), the chromatograms of test solutions exhibited a zone similar in hR_F and color to the zone due to eupatorin-5-methylether. This presence was also confirmed by comparing the UV spectra of standard and those of zones at the same position in the samples (E-H). Concerning the zone corresponding to tangeretin, the chromatograms of test solutions do not seem to have a zone at similar hR_F (A). The image under UV 366 nm after derivatization A (D) shows tangeretin as a faint blue fluorescence which is not seen in test solutions at this position. The UV spectra of relevant positions in the test solutions confirm that tangeretin cannot be identified in the three samples of Java tea (I-L).



3

Eupatorin-5-methylether (track 1) and tangeretin (track 2) in 3 samples of Java tea (track 3 and 4: Orthosiphon aristatus (Blume) Miq; track 5: Java Tea documented under UV 254 nm (A), under UV 366 nm (B), under UV 366 nm after derivatization with natural product reagent (C), under UV 366 nm after derivatization with anisaldehyde in addition (D), UV spectra of eupatorin-5-methylether (E), and comparison with the corresponding zones in the three samples (F-H), as well as tangeritin (I), and comparison to the corresponding zones in the three samples (J-L)

HPTLC-MS allows a further confirmation of determined substances. The presence of eupatorin-5-methylether and the absence of tangeretin in the 3 samples could be confirmed by mass spectrometry.



4

HPTLC-MS spectra of eupatorin-5-methylether and tangeretin and comparison with the corresponding zones in the samples

Conclusion

Together with the already established sinensetin, scutellarein tetramethylether, eupatorin, and eupatorin-5-methylether have been determined as specific markers suitable for the identification of *Orthosiphon aristatus* (Blume) Miq. by HPTLC. The presence of these four markers in the flavonoid profile is specific for Java tea.

Further information is available on request from the authors.

[1] Y. Tezuka *et al.*, Chem Pharm Bull 48 (2000) 1711–1719

[2] G.A. Akowuah *et al.*, Food Chem 87 (2004), 559–566

[3] W. Sumaryono *et al.*, Planta Medica 57 (1991) 176–180

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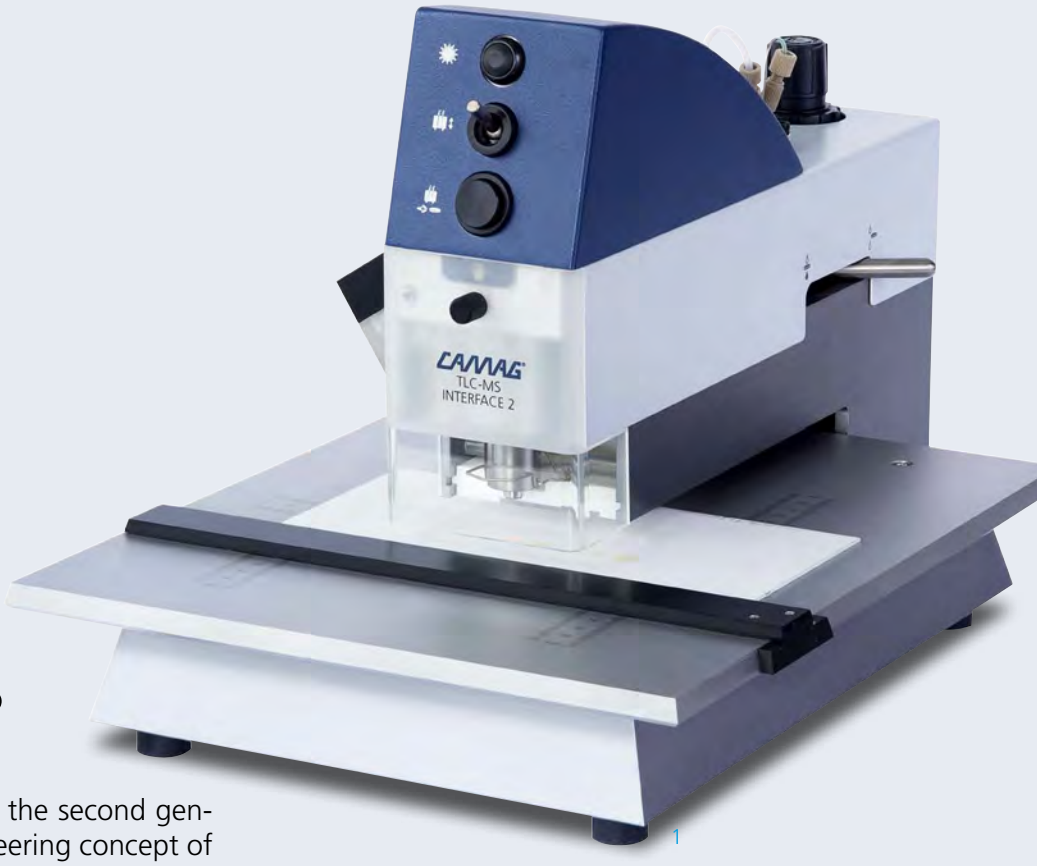
Tiên Do, CAMAG, Sonnenmattstrasse 11, 4132 Muttenz, Switzerland, tien.do@camag.com

NEW

CAMAG TLC-MS Interface 2

Identification and confirmation of substances in research and routine analysis by hyphenation of HPTLC with MS

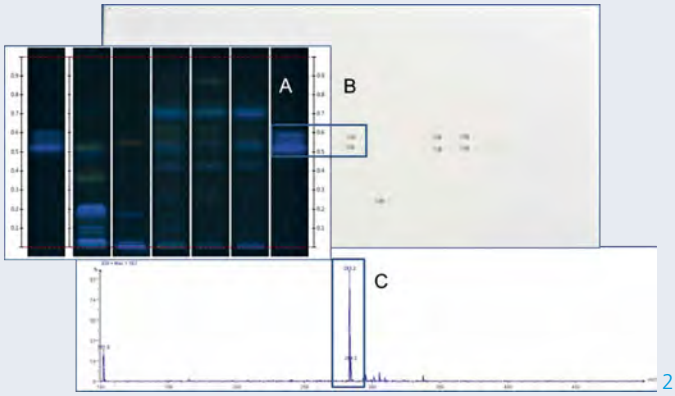


1

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 almost 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 can be obtained within a minute.

Application Example



2

Identification of ergoline psychedelics during chemical synthesis and purification (see p. 11 in this CBS issue)

A: Chromatogram of LSZ synthesis samples under UV 366 nm for localizing the zones

B: HPTLC plate after elution of zones with the TLC-MS Interface 2

C: HPTLC-ESI-MS spectra of LSZ, m/z 283.2 $[M+Na]^+$

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

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