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HPTLC is indispensible
Not only for screening of natural products

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Column: Know CAMAG

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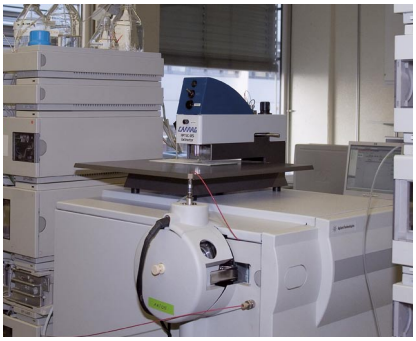
Front page: Branching sponge (Axinella canabina)
Photo: Andrej Jaklin, Rovinj, Croatia

The new TLC-MS Interface


1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Left to right: Dr. Matthias Loppacher, Head R & D CAMAG, Rolf Rolli, CEO CAMAG

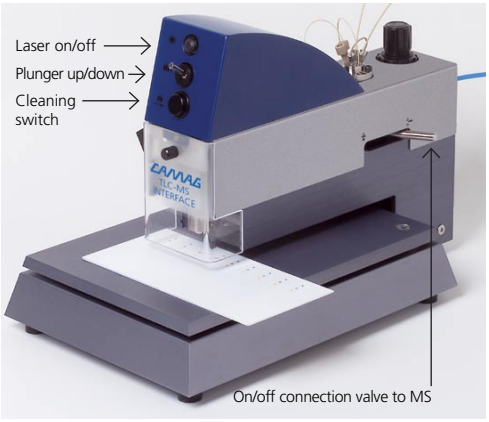
For more than 20 years efforts have been made to hyphenate TLC with mass spectrometry (MS), similar to that of HPLC and MS. Dr Luftmann, Head of the Mass Spectrometry Department at the Institute of Organic Chemistry of the University of Münster, Germany, developed an interface (ChromeXtractor) which allows such TLC-MS hyphenation [1, 2]. Dr. Morlock, assistant professor at the Institute of Food Chemistry of the University of Hohenheim in Stuttgart, Germany, modified ChromeXtractor and demonstrated the performance of this versatile interface in comparison to other technical solutions for hyphenation [2-16].

Customer surveys conducted by CAMAG in 2007 indicated a great interest in a standardized solution which initially would be limited to semi-automatic operation. Hence an appropriate commercial instrument was developed and is now being launched by CAMAG as the TLC-MS Interface. In CBS issues 93, 94, 96, 98, 100 and 101 we illustrated the mode of operation and applications of the interface. The interface has the advantage that without modification it can be integrated into any HPLC-MS system featuring atmospheric pressure chemical ionization (APCI), atmospheric pressure photoionisation (APPI), or electrospray ionization (ESI). With two fittings the interface is connected to the HPLC pump and the mass spectrometer. The substance of interest is eluted directly from the TLC/HPTLC plate and is transferred online into the mass spectrometer. Within a minute the mass spectrum is obtained.

The extraction principle

Component mixtures, even with heavy matrix load, can be separated cost-efficiently on TLC/HPTLC plates or aluminum foils. If the target zone is not visible, it can be marked either under UV 254 nm or UV 366 nm, by extrapolation of the adjacent zone made visible by derivatization, or by using the hR_F-value obtained by TLC Scanner 3. By means of a laser cross-hairs the zone to be extracted is positioned exactly under the extraction piston of the interface. The TLC-MS Interface is operated in semi-automatic mode, which means that after manual positioning of the zone the piston is lowered at the push of a button. Moving a lever starts the solvent flow through the layer and extracts the zone. Previously data acquisition has to

be started by e.g. flow injection analysis (FIA), direct flow infusion, placebo injection, or the direct data acquisition window. Afterwards the cleaning procedure is run (approx. 5 s) and the TLC-MS Interface is ready for the next analysis.

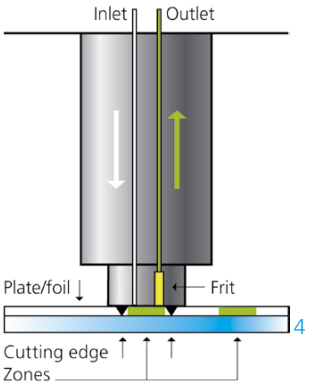


3

Interface functions and positioning the zone under the laser crosshairs.

Extraction piston

The current extraction piston has a diameter of 4 mm. Oval extraction pistons for band-shaped zones will be available. The extraction piston is lowered onto the layer with a force of approx. 20 kg thus completely sealing the zone to be extracted. Then, a suitable solvent, e.g. methanol or a mixture of methanol with ammonium formate buffer (10 mM, pH 4) 95:5 (v/v), is pumped through the inlet capillary of the piston. Typical flow rates of the HPLC pump are between 0.05 and 0.5 mL/min, preferably 0.1 mL/min. The solvent is pumped through the layer and elutes the substance. The outlet capillary is fitted with a frit to prevent contamination of the mass spectrometer with silica gel particles washed out.



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Scheme of the extraction piston


5a

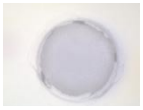
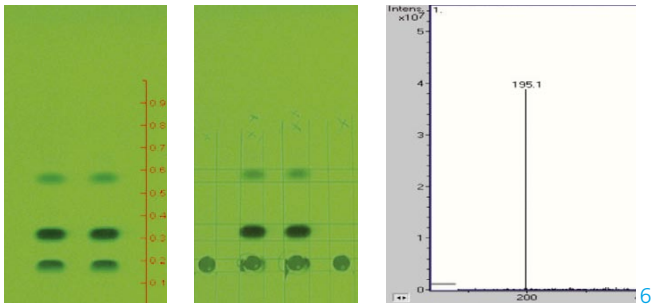

5b

Plate detail of blue dye zone before and after extraction

Example

For identification of the zone at hR_F 15 in a standard mixture of caffeine, paracetamol and acetylsalicylic acid the mass spectrum of the zone is recorded. At the same position a background spectrum of the plate is recorded and subtracted from the substance spectrum. This leads to a mass spectrum free from system peaks showing mainly substance signals – here the mass signal *m/z* 195 $[M+H]^+$ for caffeine.



6

Left: Chromatogram with 4 mm bands, middle: Same plate after extraction of zone at hR_F 15, right: Extracted zone identified as caffeine based on the mass signal at m/z 195

Further reading

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Screening for bioactive natural products in sponges

7 *Anne Klöppel and Andrea Kolm*

8 *Prof. Dr. Franz Brümmer*

The research group of Prof. Dr. Franz Brümmer[1], Biological Institute, Universität Stuttgart, works in the field of bioactive natural products from sponges (Porifera). Thereby, the focus is on the chemical ecology and the determination of novel substances with potential pharmacologic properties. The cultivation (*in situ, ex situ* and *in vitro*) and depending changes in the metabolite spectrum of certain sponge species is a further aspect.

In collaboration with Dr. Gerda Morlock[2], Institute of Food Chemistry, University of Hohenheim, culture related variances in compound pattern and bioactivity were analysed by HPTLC, bioactivity assay and mass spectrometry. This coupling is particularly suitable for drug discovery and was used in the field of bioactive natural products for the first time.

Introduction

Marine invertebrates belong to the most efficient sources for bioactive compounds with pharmaceutical properties. There are about 800 novel substances discovered every year, of which 45 % are provided by sponges. As sessile filter feeders lacking morphological protective mechanisms, sponges use these compounds to combat feeding pressure, neighbourly competition and biofouling. The spectrum of activity shows antibacterial, anti-inflammatory, cytotoxic and virostatic properties. Despite the large diversity of secondary metabolites only a few compounds have entered the clinical market, e.g. Ara A®, 9-β-D-arabino-furanosyladenine from *Cryptotethya crypta* which exhibits antiviral properties against the *Herpes simplex*-Virus.

Before entering the pharmaceutical market or clinical tests, an extensive combination of different analyses is needed. First, general bioactivity of crude extracts is determined by standard bioassays. Examples are the agar disc diffusion assay with standard strains, e.g. *Escherichia coli* and *Bacillus subtilis*, or the luminescent bacteria assay based on *Vibrio fischeri*. However, to show a clear correlation between single substances and bioactivity a bioassay directed fractionation is needed. For isolation and purification different chromatographic systems (e.g. solid phase extraction, gel permeation chromatography, semipreparative HPLC) have to be connected. This is followed by further bioactivity screening of any single compound or fraction. Established combinations for structure elucidation are HPLC-MS and NMR.

In extensive search and identification of novel bioactive compounds, HPTLC provides an abbreviated, more robust (little matrix effects) and economic analysis compared to HPLC. Parallel chromatographic separation of 30 sponge extracts is possible. The combination with a bioassay based on the luminescent bacteria assay with *Vibrio fischeri* (DIN EN 11348) and following structure elucidation via high resolution mass spectrometry provides an effective system for metabolite and bioactivity screening in sponges [1]. Substances of interest can be extracted directly from the plate and transferred to the MS system without time and cost intensive isolation and purification processes. Within a minute respective mass signals were obtained and

molecular formulae by high resolution MS. This method shows detectability comparable to HPLC-MS due to the complete extraction of substance bands from the HPTLC plate.

Sample preparation

After sampling and cultivation (*in situ* and *in vitro*) 1–5 cm³ of each specimen were frozen in liquid nitrogen and lyophilised. The samples were powdered and 100 mg each were weighed out into sterile reaction tubes. The extraction was done with 10 mL high purity methanol using a shaker for 20 hours. After centrifugation 2 mL of the supernatant were used.

Standard solutions

Avarol and avarone (isolated from *Dysidea avara* by Prof. Dr. Werner Müller, University of Mainz) were dissolved in high purity methanol (0.1 mg/mL each).

Layer

HPTLC plates silica gel 60 F_{254} (Merck), 20 x 10 cm, prewashed by developing in methanol followed by drying on the TLC Plate Heater at 100 °C for 15 min.

Sample application

Bandwise with the Automatic TLC Sampler 4, band length 4 mm, track distance 6 mm, distance from lower edge 8 mm, distance from side 10 mm, application volumes of 20 µL for extracts and 0.2–2 µL for standards (20–200 ng/band).

Chromatography

In AMD 2 system using a 15 step gradient based on methanol, dichloromethane and n-hexane. The separation was performed under nitrogen within 2.5 h and a final migration distance of 53 mm.

Bioactivity based detection

HPTLC plates were documented by use of the Digi-Store 2 System at 254 nm, 366 nm, and white light illumination (reflectance). For bioactivity screening plates were automatically dipped, by means of the TLC Immersion Device, at a speed of 3.5 cm/s and with an immersion time of 1 s into the luminescent

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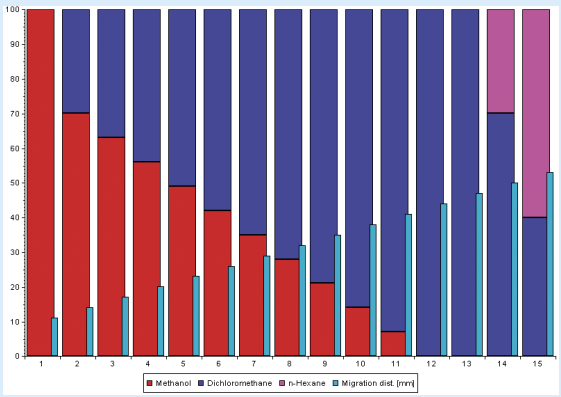
CAMAG AMD 2 System
(Automated Multiple Development)

The CAMAG AMD procedure can be successfully employed for reproducible gradient elution with silica gel as the stationary phase. It can be utilized for the separation of complex and matrix loaded samples with a resolution that cannot be achieved with other developing techniques.

Here the AMD technique is employed for the screening of natural sponges for bioactive ingredients. Focus is laid on standardising a procedure to characterize a large number of different sponges independent of their matrix content, rather than exploiting the optimum resolution attainable with the AMD method.

The universal gradient depicted below was used for development. The fact that overloading of some tracks was unavoidable is immaterial for the analytical task given.



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bacteria (*Vibrio fischeri*) suspension (BioLuminex assay, ChromaDex, Boulder, CO, USA). Bioactive compounds led to an inhibition or enhancement of luminescence intensity documented with the BioLuminizer (exposure time 30 s). For digital quantification the images can be imported into VideoScan.

Coupling with ESI-MS

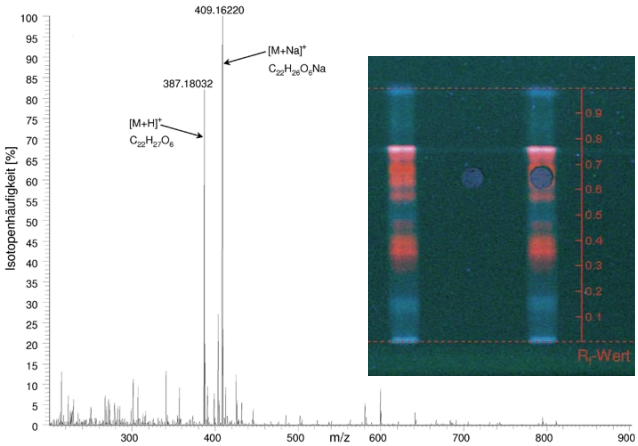
Zones of interest were marked on the plate and transferred via the online extractor (Chromextraktor, ChromAn) into the ESI-MSD (Agilent; capillary voltage 4 kV, desolvation temperature 300 °C, drying gas 10 L/min, nebulising gas 30 psig) by means of methanol – ammonium formiate buffer (10 mmol/L, pH 4) 95:5 with a flow rate of 0.1 mL/min. Mass spectra were acquired in the positive full scan mode between *m/z* 200 and 900 and confirmed using high resolution MS (LTQ Orbitrap XL hybrid FT-MS, Thermo Fisher Scientific).

Results and discussion

The *in vitro* cultivation of sponge cells, so-called primmorphs (proliferating 3D-cell aggregates), may be a possibility for producing sponge biomass and therewith bioactive compounds on a large scale.

The synthesis of interesting secondary metabolites was continued in cell culture which could be demonstrated by HPTLC-bioactivity screening. However, some sponges (e.g. *Axinella polypoides*) showed differences in qualitative compound composition, although there was no influence on the overall toxicity of sponge extracts. Often, extracts obtained from in vitro cultured sponges (e.g. *Axinella polypoides*, *Suberites domuncula*, *Petrosia ficiformis*) led to a stronger inhibition of luminescence concerning the same substance from equal concentrated in situ extracts. This might be due to a stress related increase of metabolite synthesis. Also some species accumulated further possible bioactive compounds lacking in the metabolite patterns from *in situ* conditions (e.g. *Suberites domuncula*). Thus, *Dysidea avara* produced a substance during in vitro cultivation which induced a luminescence enhancement of bacteria. One further *Vibrio fischeri*-toxic metabolite was missing or synthesised in small amounts only. A possible involvement of symbiotic cyanobacteria in product synthesis was examined in extracts from *Petrosia ficiformis*. There was a modification in qualitative metabolite pattern but not in overall bioactivity.

The coupling of HPTLC, Bioluminex assay and mass spectrometry enabled the very fast and effective separation of 30 different sponge extracts in parallel, the determination of bioactive compounds and their structure elucidation. This application led to the first time detection of a bioactive substance produced by a freshwater sponge (*Ephydatia fluviatilis*) which, however, was not synthesised in *in vitro* cultivation. Via coupling to high resolution mass spectrometry the exact monoisotopic mass of the protonated molecule as well as the sodium adduct was determined with *m/z* 387.18032 $[M+H]^+$ and *m/z* 409.1622 $[M+Na]^+$, respectively, leading to a molecular formula of $C_{22}H_{27}O_6$.



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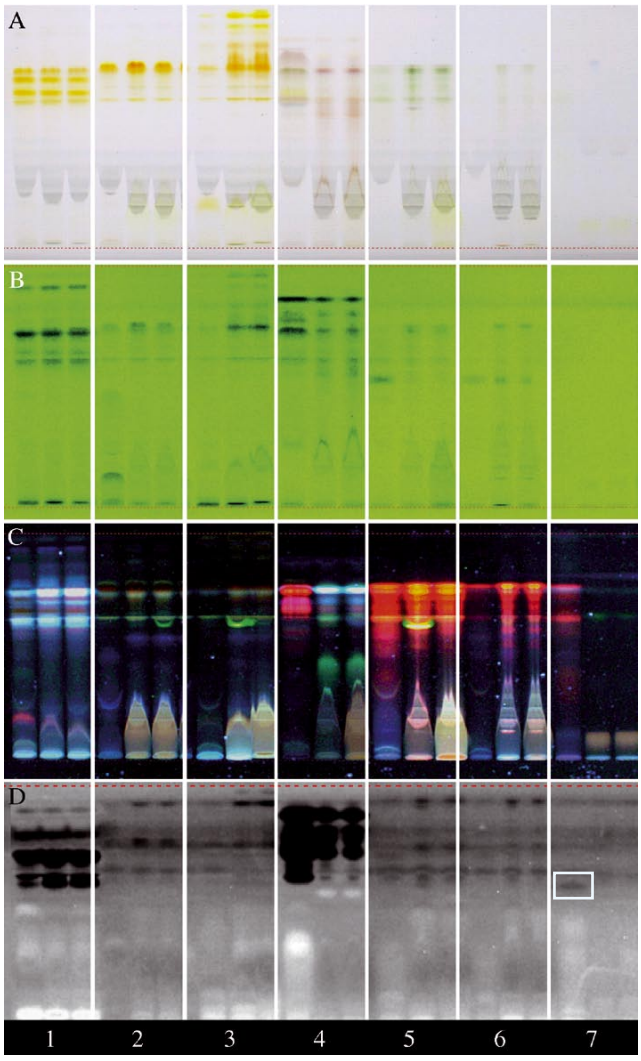
Left: HPTLC-ESI-MS-spectrum (positive-ion mode) of a bioactive zone from the freshwater sponge Ephydatia fluviatilis leading to the monoisotopic mass of the protonated molecule with m/z 387.18032 $[M+H]^+$ and a mass divergence of 0.7 ppm.

Right: Image detail of a developed HPTLC plate documented at UV 366 nm: the bioactive substance (hR_F 65) was extracted via the extraction interface (Ø 4 mm) (left track: before extraction, middle: blank value, right track: after extraction).



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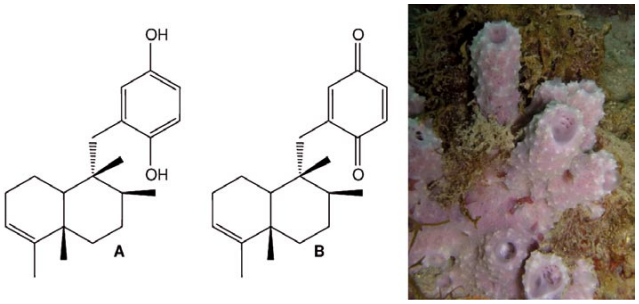
HPTLC plate detected under (A) white light, (B) at 254 nm, (C) at 366 nm and (D) via BioLuminizer. Detailed plate images 1–7 show in situ sponge extracts on left track and respective primmorphs (in vitro) on middle (cell culture medium according to Le Pennec et al. (2003) and right track (cell culture medium according to Zucht (2005)): Acanthella acuta (1), Axinella polypoides (2), Suberites domuncula (3), Dysidea avara (4), Petrosia ficiformis with symbiotic cyanobacteria (5), Petrosia ficiformis without symbiotic cyanobacteria (6), Ephydatia fluviatilis (7) here: cell culture medium according to Harsha (1983).

Inhibition of bioluminescence (black zone), enhancement of bioluminescence (white zone), unknown bioactive compound from Ephydatia fluviatilis (white box).

Already known bioactive natural products are the antiviral and cytotoxic metabolites avarol and avarone. The detection limits (LOD) of bioactivity were 70 (avarol) and 60 ng/band (avarone) which is considered highly satisfying for the screening of sponges.



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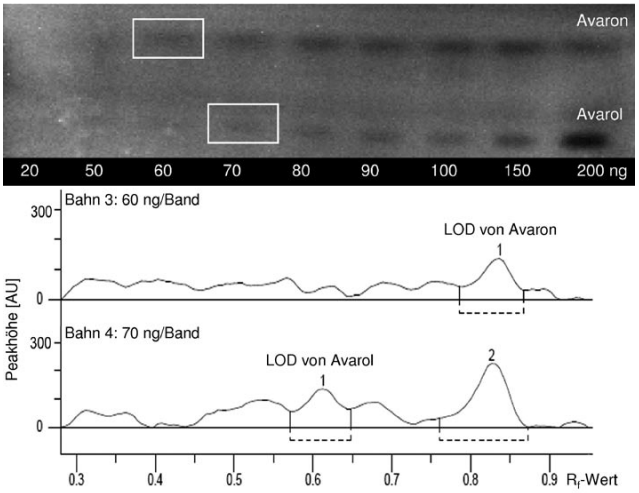
Structure formulae of avarol (A) and avarone (B) from Dysidea avara (right). Presently, avarol is applied in ointments against psoriasis (International Patent Application DE 1991-4137093). Visual (white box) and digital detection limit (via VideoScan) of avarone (60 ng/band) and avarol (70 ng/band) after HPTLC-bioluminescence detection.



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Visual (white box) and digital detection limit (via VideoScan) of avarone (60 ng/band) and avarol (70 ng/band) after HPTLC-bioluminescence detection.

This effect based analysis also enables the detection of UV-inactive bioactive metabolites not visible with prevalent detectors like HPLC-DAD. One further advantage is the elimination of time intense isolation and purification processes with reference to HPLC-MS. Due to the evaporation of solvents after the chromatographic separation, there is no influence on the detection by inactivation of enzymes or living organisms like bioluminescent bacteria.

[1] A. Klöppel, W. Grasse, F. Brümmer, G. Morlock, J. Planar Chromatogr. 21 (2008) 431-436

[1]Prof. Dr. Franz Brümmer, Institute of Biology, Department of Zoology, Universität Stuttgart, Pfaffenwaldring 56, 70569 Stuttgart, Germany, franz.bruemmer@bio.uni-stuttgart.de

[2]PD Dr. Gerda Morlock, Institute of Food Chemistry, University of Hohenheim, Garbenstrasse 28, 70599 Stuttgart, gmorlock@uni-hohenheim.de

Online coupling of HPTLC-MS will be our Hot Topic in 2009!

Hyphenating TLC/HPTLC with mass spectrometry appears to hold considerable promise for those analysts who previous have had reservations towards the use of Planar Chromatography. The hyphenation opens a new dimension for the technique and for some, it may make it more prestigious from the scientific view.

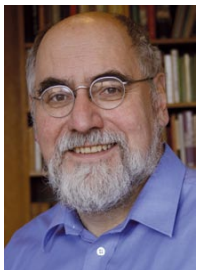
The new interface that is required for this hyphenation is featured on page 16 of the white pages, and its application is reported on pages 2/3. More applications had been described already in some recent CBS issues.

This year CAMAG will be running a campaign of one-day seminars in four German cities to acquaint interested analysts in the method and its potential. In the morning session scientists, who have been involved in the development of the interface, and, who have already collected experience with the method, will give lectures. In the afternoon the technique will be demonstrated in the laboratory with samples brought by the participants.

Seminars will be held at:

26.03.2009	Zweckverband Landeswasserversorgung, D-89129 Langenau
30.04.2009	University of Applied Science D-77652 Offenburg
26.06.2009	University of Münster D-48140 Münster
01.10.2009	Technical University D-13355 Berlin

If you are interested in online coupling of HPTLC-MS or if you know others in your company who might have interest, please let us know at marcel.hug@camag.com. We will have your CAMAG distributor contact you and will let you know of any seminars on this subject in your area.



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Dr. Heinrich Luftmann, Head of the Mass Spectrometry department at the Westphalian Wilhelms University of Münster, luftman@uni-muenster.de

Field of interest: analysis of samples from a wide variety (>18000 per year), e.g. synthesis products, organometallic compounds, carbohydrates, peptides, polymers; development of instruments and peripherals for mass spectrometry

Lecture: ***TLC-MS in organic syntheses***

16

Dr. Wolfgang Schulz, Head of the Laboratory for Special Analyses for Routine and Research, Zweckverband Landeswasserversorgung in Langenau, Schulz.W@lw-online.de – lecturer at University of Applied Science of Aalen for many years

Field of Interest: non-target screening using planar chromatography, HPLC, MS and bio-activity based detection

Lecture: ***Use of HPTLC-MS coupling for identification of organic trace substances in raw and drinking water***

17

Prof. Dr. Hans-Rudolf Schmutz, University of Applied Science of Life Sciences Nordwestschweiz in Muttenz, hansrudolf.schmutz@fhnw.ch – teaching classical and instrumental analysis

Field of Interest: pharmaceutical analysis using different separation methods, particularly hyphenated techniques

Lecture: ***HPTLC-MS of pharmaceutical compounds and plant ingredients***

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Assist. Prof. Dr. Gerda Morlock, University of Hohenheim in Stuttgart, gmorlock@uni-hohenheim.de – teaching instrumental analysis and food analysis

Field of interest: Online coupling HPTLC-MS, bio-activity based analysis, application by printers, nano-structured layers, and digital evaluation of planar chromatograms

Lecture: ***HPTLC-MS in food and pharmaceutical analysis***