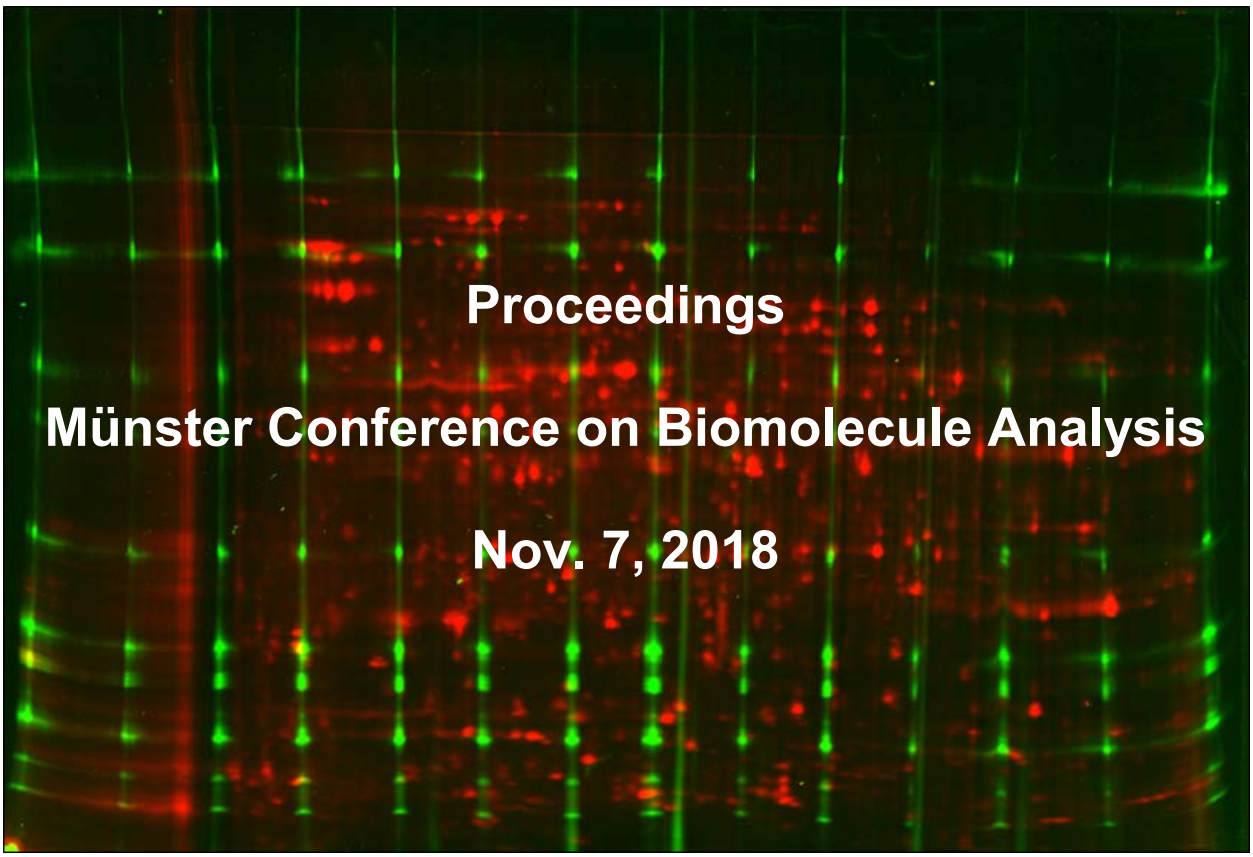

# Proceedings

# Münster Conference on Biomolecule Analysis

## Nov. 7, 2018

Organized by the Core Unit Proteomics
of the Interdisciplinary Center for Clinical Research Münster
Prof. Simone König & Dr. Rita Naskar

# Preface

The Münster Conference on Biomolecule Analysis 2018 focused on thin-layer chromatography (TLC) in conjunction with mass spectrometry (MS). In a special workshop on TLC-MS, organized by the companies Waters, Andrew Alliance, MSC Consult, Camag and Merck, attendees could watch live experiments and see instruments in action. A historical overview about TLC was given by Teresa Kowalska, an expert in the field and author of a book series on the topic. Furthermore, the inventor of the TLC-extractor, Heinrich Luftmann, discussed the development of his device, which has been commercialized. High-ranking speakers from the TLC field reported about their application of the technique in their research. The conference also provided a platform for companies to showcase their products and interact with customers in workshops. The international event successfully met the increasing interest in protein analysis technologies and provided a valuable information source in particular for Ph.D. students.

Since 2004, the Core Unit Proteomics (CUP) of the Interdisciplinary Center for Clinical Research Münster has organized the event as an annual series of bioanalytical conferences.

Münster, Nov. 19, 2018
*Simone König*

# Poster Abstracts

## Organization analyses of the type I secretion system of *Salmonella enterica*

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The cooperative function of two protein secretion systems is required for adhesion to and invasion of polarized epithelial cells by *S. enterica* serovar Typhimurium. Before the *Salmonella* Pathogenicity Island 1-endoced type III secretion system (SPI1-T3SS) mediates invasion, the giant adhesin SiiE, the only known substrate of the SPI4-encoded type I secretion system (SPI4-T1SS), allows adhesion to the apical side of polarized host cells.
The SPI4-T1SS is composed of the canonical subunits SiiF (ATPase), SiiD (periplasmic adapter protein) and SiiC (outer membrane pore). SiiE as the substrate mediates the adhesion of *Salmonella* to polarized epithelial cells and enables the secretion of T3SS-effector proteins which leads to an efficient invasion in polarized cells. In contrast to well-studied type I secretion systems of other bacteria, the SPI4-T1SS also features two non-canonical subunits SiiA and SiiB. We suggest that these subunits form a proton conducting channel whose proton motive force is linked to the release of SiiE.
So far, the stoichiometry of the SPI4-T1SS and the function of the non-canonical subunits SiiA and SiiB are not completely understood.
We investigated the C-terminal domains of SiiA and MotB, which showed significant similarities in the protein structure. In several functional experiments, we attained further characterized of these domains and underpinned the importance of these C-terms.
Beside this, we set out to purify the important virulence factor SPI4-T1SS in its complex form. Enriched T1SS will be analysed by mass spectrometry and single particle cryo-TEM to make out the organisation and the stoichiometry of the T1SS. These further characterizations will lead to an improved understanding of the *Salmonella* Typhimurium invasion strategy and could point out new targets in the fight against bacterial infections.

Gerlach *et al.* Cooperation of *Salmonella* pathogenicity islands 1 and 4 is required to breach epithelial barriers (2008), http://doi.10.1111/j.1462-5822.2008.01218.x
Wille *et al.* SiiA and SiiB are novel type I secretion system subunits controlling SPI4-mediated adhesion of *Salmonella enterica* (2014), http://doi.org/10.1111/cmi.12222
Du *et al.* Structure of the AcrAB-TolC multidrug efflux pump (2014), http://doi.org/10.1038/nature13205
Hizukuri *et al.* The peptidoglycan-binding (PGB) Domain of the *Escherichia coli* Pal Protein can also Function as the PGB Domain in *E. coli* Flagellar Motor Protein MotB (2009), http://doi.org/10.1093/jb/mvp061
SiiA shows significant similarities in structure and function with well-studied proteins like MotB in *Escherichia coli*. Both proteins exhibit α-helices at the C-terminus which indicate in MotB a peptidoglycan binding site.

## Isoflavonoids with inhibiting effects on human Hyaluronidase-1 and norneolignan clitorienolactone B from *Ononis spinosa* L. root extract

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Human hyaluronidase-1 (Hyal-1) is one of the main enzymes in the homeostasis of hyaluronic acid (HA), the main polysaccharide of extracellular matrix. Development of specific Hyal-1 inhibitors might be a promising target for improved wound healing, tissue regeneration, and looking at renal function for diuresis. By using surface-displayed Hyal-1 on *Escherichia coli* F470 cells, HA as substrate and stains-all method for quantification of undegraded HA, the respective enzyme activity can be determined easily. Based on the traditional use of extracts from the roots from *Ononis spinosa* L. (Restharrow root) as a weak diuretic to achieve flushing of the urinary tract and as an adjuvant in minor urinary complaints the herbal material was selected for bioactivity guided fractionation for compounds with Hyal-1 inhibition activity. Hot water and hydroalcoholic extracts showed moderate inhibiting effects ($IC_{50}$ 1.36 resp. 0.73 mg/mL) while dichloromethane extract exerted an $IC_{50}$ of 190 µg/mL. Bioassay guided fractionation of the dichloromethane extract yielded four isoflavonoids with anti Hyal-1 activity: onogenin **1**, sativanone **2**, medicarpin **3** and calycosin-D **4** with inhibition rates of 25.4, 61.2, 22.4 and 23.0 %, respectively at test concentration level of 250 µM. The norneolignan clitorienolactone B **5**, the first time described for the genus *Ononis*, was inactive. The $IC_{50}$ of sativanone, the most active compound was determined with 151 µM, which was better than that of the positive control glycyrrhizinic acid (177 µM). Thus, a possible explanation for diuretic properties of *Ononis spinosa* L. root extract may be postulated from the results so far obtained.

## TLC-MS coupling to elucidate the lipid composition of lipid mixtures

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Matrix-assisted laser desorption ionization (MALDI) and electrospray ionization mass spectrometry (ESI MS) are frequently used in lipidomics to elucidate the lipid compositions of different samples. However, some (phospho)lipid species ionize better than others and therefore, ionization-dependent MS methods, such as MALDI and ESI MS suffer from ion suppression. A separation of lipid classes prior to MS by thin layer chromatography (TLC) can help to overcome this effect. Thus, the lipid composition of a distinct sample can be unraveled, at least quantitatively, by coupling TLC and MS. The knowledge about the lipid composition is extremely important because distinct (phospho)lipids act as second messengers and/or are well-known markers for different diseases.
Next to our methodological approaches we will show analyses of different samples ranging from biological samples to artificial lipid mixtures and their oxidation products and present the usefulness of TLC-MS coupling.

## A combination of thin-layer chromatography, antibody-overlay detection and desorption electrospray ionisation Fourier-transform-ion cyclotron resonance mass spectrometry provides insights in lectin carbohydrate-binding specificity

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Lectins acting as carbohydrate recognition determinants in diverse biological processes are widely used in a broad range of applications including the detection, isolation and structural studies of glycoconjugates due to their specific sugar-binding properties. In the present study the carbohydrate binding specificity of a lectin previously isolated from the seeds of *Trichosanthes dioica* (TDSL) [1] was probed comprehensively. To this end, we made use of a combination of immunodetection of glycosphingolipids (GSLs) [2] acting as high affinity ligands of TDSL and their subsequent accurate and precise analysis by use of desorption electrospray ionisation Fourier-transform-ion cyclotron resonance mass spectrometry (DESI FT-ICR MS). Binding specificity of TDSL towards GSLs was investigated by thin-layer chromatography (TLC) overlay assays with GSLs from different sources employing an antibody directed against xylose present in pauci-mannose type *N*-glycans found in TDSL.
For DESI FT-ICR MS measurements, the plate was placed onto a movable sample stage and analytes were desorbed and ionised directly from the plate by use of methanol as a spray solvent. Mass analysis was performed with a Bruker Apex II FT ICR mass spectrometer equipped with a 7 T magnet.
Previous studies suggested a preferred binding of TDSL towards galactose and/or *N*-acetylgalactosamine [1]. Here, we show that careful analysis of the carbohydrate-binding specificity of TDSL exhibits strong binding to gangliosides comprising α2-6-linked sialic acid as well as weaker interaction with the Galβ1-4GlcNAc motif present in neutral GSLs. Noteably, gangliosides harbouring terminally α2-3-linked sialic acid were not recognized by TDSL. Immunostained bands were analysed by DESI FT-ICR MS and lectin-binding GSLs could be desorbed and ionised directly from the TLC plate without interferences from the lectin, antibodies or the staining reagent. Positive ion mode spectra showed the formation of stable, doubly sodiated molecules which allowed for unambiguous identification of high and low affinity ligands of TDSL. These results indicate that TDSL has to be regarded as a lectin with preferential binding specificity towards oligosaccharides containing terminally α2-6-linked sialic acid and minor affinity towards terminal Galβ1-4GlcNAc moieties. The present study demonstrates the potential of the hyphenation of planar chromatographic separation and formation of gaseous ions directly from the surface of the TLC plate by DESI for the analysis of immunostained GSLs serving as lectin ligands. Knowledge of carbohydrate-binding specificities of lectins is a prerequisite for their use in biomedical applications e.g. as probes for mammalian ganglioside receptors of influenza A viruses.

[1] N. A. M. Sultan et al. Arch. Biochem. Biophys. 2004, 432, 212–21.
[2] I. Meisen et al., Biochim. Biophys. Acta 2011, 1811, 875–896.

## Chiral amino acid analysis for the detection of D-proline in hypertrehalosaemic neuropeptides of cicadas

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For more than two decades, it has been known that the neurosecretory glands of the cicadas, the corpora cardiaca (CC), synthesize two isobaric peptides with hypertrehalosaemic activity. Both decapeptides have the same amino acid sequence (pGlu-Val-Asn-Phe-Ser-Pro-Ser-Trp-Gly-Asn-$NH_2$) but differ in retention time in reversed-phase liquid chromatography. A synthetic peptide with the above sequence co-eluted with the second, more hydrophobic peptide peak of the natural cicada CC extract. The modification associated with the less-hydrophobic peptide was not clear. Ion mobility separation, which is sensitive to changes in conformation, in conjunction with high-resolution mass spectrometry (nanoUPLC, Synapt G2 Si, Waters Corp.), was used to investigate this phenomenon in *Platypleura capensis*. Different drift times in buffer gas for both the intact peptides and some of their fragment ions were detected. Based on the ion mobility and fragment ion intensity of the corresponding ions it was shown that the region $Pro^6$-$Ser^7$-$Trp^8$ contained a different structural feature to that of the L-amino acids present in the known peptide [1]. The chromatographic behavior of synthetic peptides excluded the presence of D-Ser and D-Trp in these positions, whereas the synthetic peptide containing D-Pro had the same retention time as the less hydrophobic natural peptide [2,3]. We have employed acid hydrolysis and derivatisation of the resulting amino acids with a variant of Marfey's reagent [4] in order to further investigate the presence of D-proline in the unknown peptide hormone.

[1] König S, Marco H, Gäde G.; *Anal Bioanal Chem.;* **2017**; *409*: 6415.
[2] König S, Marco H, Gäde G, Does the presence of D/L-proline in the isobaric hypertrehalosaemic neuropeptides of cicadas explain their isomerism? 51th DGMS, Saarbrücken, March 2018.
[3] König S, Marco H, Gäde G. D-Proline – Comment to "An overview on D-amino acids". *Amino Acids;* **2017**; *50*: 359.
[4] Bhushan R., Kumar, R.; *Anal Bioanal Chem*; **2009**; *394*:1697-1705.

## Monitoring α-MSH degradation by prolylcarboxypeptidase in conditioned media of human dermal fibroblasts

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Prolylcarboxypeptidase (PRCP) is s serine protease that regulates a number of hormonal pathways including the proopiomelanocortin system. It metabolizes α-MSH ($_{Ac}$SYSMEHFRWGKPV$_{NH2}$) into biologically inactive α-MSH (1-12) by the removal of the N-terminal valine. α-MSH (1-13) has a variety of biological functions in the skin, such as regulation of pigment formation, exocrine activity, inflammatory reactions and immunomodulation. As α-MSH has strong anti-inflammatory and immunomodulatory actions we hypothesized that PRCP due to deactivation of the biological activity of α-MSH could play role in the pathogenesis of the immune-mediated skin diseases. The expression of PRCP in various cutaneous cell types at RNA and protein level in vitro was examined. PRCP transcripts were detected in normal human melanocytes, normal human keratinocytes and human dermal fibroblasts (HDFs) from different donors as shown by endpoint reverse transcriptase- polymerase chain reaction (RT-PCR). Western immunoblotting revealed expression of PRCP protein, however, only in HDFs. Here, PRCP could be visualized as a granular cytoplasmic staining as shown by immunofluorescence analysis.
A method was developed to detect the enzymatic cleavage product directly. To that end, nano-liquid chromatography coupled to high-resolution mass spectrometry was programmed to target the triply-charged peptide ions. Cell supernatants were purified for the purpose by aceton precipitation followed by solid-phase extraction. α-MSH (1-12) was formed by HDFs treated with α-MSH in conditioned media suggesting expression and secretion of active PRCP. Quantitative real-time RT-PCR analysis further revealed a time-dependent upregulatory effect of both tumor necrosis factor and ultraviolet A irradiation but not interleukin-1β on PRCP mRNA in HDFs. However, Western immunoblotting of total cell lysates and detection of PRCP in cell culture supernatants employing ELISA did not reveal upregulation of this enzyme in HDFs exposed to these stressors. Immunohistochemical studies are currently underway to determine the expression pattern of PRCP in normal and diseased human skin *in situ*. In sum, our data provide first evidence of cell type-specific expression of PRCP in human skin types. Further studies have to assess the relevance of these findings for cutaneous biology.

## Workflow for the Analysis of Comparative Fluorescence Gel Electrophoresis Experiments Using Delta2D

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Comparative fluorescence gel electrophoresis (CoFGE) was developed for the reproducible assignment of protein spot coordinates after two-dimensional gel electrophoresis (2D –PAGE, [1-5]). By overlaying traditional 2D-PAGE with a 1D-PAGE reference channel it is possible to correct for gel-to-gel variation. The method was set up for singular samples and is thus complimentary to differential gel electrophoresis (DIGE), which uses replicates. Precast gels were commercialized by SERVA Electrophoresis (Mercator-Gels). No software product was available for the analysis of CoFGE experiments so that the software developed by the German company DECODON (Greifswald) was evaluated for the purpose.
The goal of data analysis is the export of corrected spot coordinates for the proteins of interest. The key function of Delta2D is warping, which deforms the gel images so that they can be virtually overlayed. CoFGE experiments follow a strict protocol and provide an analyte gel image along with a corresponding reference protein grid. In that way it is possible to correct large numbers of gels run over a wide period of time and even at different labs. Depending on the project design, there are different options for referencing. For projects, which continue over an indefinite period of time it is useful to generate a master gel as a fusion gel of at least three experiments performed right at the beginning of the project. This gel will then serve as a global reference. In case of smaller sets of gels within a temporary research project, one reference grid can be selected as master reference to which all other reference gels are matched. In addition, semi-quantitative information about protein amounts can be obtained by comparison to the internal standard with known concentration.
The manual for the analysis of CoFGE experiments with Delta2D is provided at urn:nbn:de:hbz:6-97129496060.

[1] S. König, W. Wang, L. Grün, D. Ackermann, Improved gel electrophoresis. EP11167383.6, May 25, 2011; WO 2012/159769 A1 Nov. 29, 2012; Patent No. 12729346.2-1554, 25.05.12, publication number EP 2 715 331
[2] D. Ackermann, S. König, Comparative 2D Fluorescence Gel Electrophoresis. In: Difference Gel Electrophoresis: Methods and Protocols. Ed. K. Ohlendieck (2017), Methods in Molecular Biology 1664, Chapter 6, pp 69-78, Humana Press by Springer Nature, New York
[3] M. Hanneken, K. Slais, S. König, pI control in comparative fluorescence two-dimensional gel electrophoresis (CoFGE) using amphoteric azo dyes. EuPa Open Proteomics 2015, 8:36-39
[4] M. Hanneken, S. König, Horizontal comparative fluorescence two-dimensional gel electrophoresis (hCoFGE) for improved spot coordinate detection. Electrophoresis 2014, 35: 1118-1121
[5] D. Ackermann, W. Wang, B. Streipert, B. Geib, L. Grün, S. König, Comparative fluorescence two-dimensional gel electrophoresis using a gel strip sandwich assembly for the simultaneous on-gel generation of a reference protein spot grid. Electrophoresis 2012, 33:1406-1410

## Determination of δ-aminolevulinic acid-induced Protoporphyrin IX in blood

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Protoporphyrin IX (PPIX) is a hydrophobic, biogenic molecule and the direct precursor of heme. All living cells are able to synthesize heme, but, nevertheless, 85% of it is produced in bone marrow and most of the rest in the liver[1]. Due to its structure and conjugated π-system PPIX absorbs light at ~400 nm - the specific absorption in region of Soret band is a common feature of all porphyrins. PPIX excitation results in two characteristic emission peaks at 635 and 704 nm[2]. In neurosurgery these properties of PPIX are used in δ-aminolevulinic acid-induced fluorescence guided resection (ALA-FGR) of glioblastoma multiforme (GBM) providing improved tumor resection. However, the aim of complete resection of malignant tissue can still not be achieved so that patients suffer from recurrent tumors[3]. It is envisaged to diagnose tumor reoccurrence and growth based on PPIX levels in blood, which would be cost-effective at lower strain for the patient.
Thus, we developed an analytical method to test the hypothesis. PPIX was extracted from anticoagulated whole blood by liquid-liquid extraction and detected with a coupling of capillary liquid chromatography to an ion trap mass spectrometer (Agilent HP1100, Esquire3000, Bruker; CapLC-IT). Our results indicate higher PPIX levels in whole blood of patients suffering from GBM, who received ALA prior to venepuncture, in comparison to a healthy person without ALA administration. A time-series of PPIX levels in whole blood of a GBM patient after ALA administration agreed with the observed fluorescence changes in tissue[4]. The maximum of the PPIX level in whole blood is reached with a two-hour delay compared to the maximum of the fluorescence intensity in GBM tissue. These first results encourage further investigations both in whole blood, where PPIX is measured as a sum parameter of δ-aminolevulinic acid-induced and endogenous PPIX, and serum, which provides the purer analyte, but much less of it.

[1] Sachar et al. Protoporphyrin IX: the Good, the Bad, and the Ugly. *J. Pharmacol. Exp. Ther.,* **2016**; 356 (2), S. 267-275.
[2] Stummer et al. Technical Principles for Protoporphyrin-IX-Fluorescence Guided Microsurgical Resection of Malignant Glioma Tissue. *Acta Neurochir.,* **1998**; 140 (10), S. 995-1000.
[3] Stepp und Stummer. 5-ALA in the management of malignant glioma. *Lasers Surg. Med.,* **2018**; 50 (5), S. 399-419.
[4] Stummer et al. In vitro and in vivo porphyrin accumulation by C6 glioma cells after exposure to 5-aminolevulinic acid. *J. Photochem. Photobiol. B,* **1998**; 45 (2-3), S. 160-169.

## Fascinating *Stachybotrys* – Novel Cytotoxic Meroterpenoids, Chemically Inspired Isolation Approaches and Application of an LC-MS/MS Multi-Method

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The genus *Stachybotrys* is commonly associated with indoor water damage and has become increasingly popular since the 1990s within cases of idiopathic pulmonary hemosiderosis in infants [1]. There are still controversial discussions, whether the fungal growth was correlated with the diseases. *Stachybotrys* produces a broad variety of secondary metabolites including macrocyclic trichothecenes, atranones and phenylspirodrimanes. In the past, investigations concerning the phenylspirodrimanes have been neglected, although they occur in even higher levels and in a remarkable multitude of different compounds. Moreover, they are known to be cytotoxic as well as immunosuppressant and additionally, combinatory effects among them have never been considered. For this purpose, a series of phenylspirodrimanes occurring in all common *Stachybotrys* species were isolated in order to be used as reference standards for the development of an LC-MS/MS quantitation multi-method. The general objective of this multi-method is to enable the determination in fungal extracts and various matrices, such as building materials, dust, feedstuff, and physiological samples. A noteworthy representative of the phenylspirodrimane family is stachybotrydial. Its structural properties led to the development of chemically inspired isolation approaches, either based on supplementation of the culture media or semisynthesis. Both strategies could potentially be utilized to simplify access to certain reference standards from *Stachybotrys*.

In the course of gaining new insights into the secondary metabolite profile, three novel cytotoxic meroterpenoids, namely stachybotrychromenes A-C, were discovered [2]. Their structures were elucidated by extensive spectroscopic analysis (NMR, HRMS, CD). First cytotoxic effects have been determined and the new compounds clearly contribute to the overall toxicity profile of this fungus.

Consequently, the genus *Stachybotrys* offers an enormous potential for future investigations to conclusively determine the role of this fungus within specific building-related case studies as described above and to clarify the relationship between human health and fungal contamination in indoor environments in general.

[1] R. A. Etzel, E. Montaña, W. G. Sorenson, G. J. Kullman, T. M. Allan, D. G. Dearborn, *Arch Pediatr Adolesc Med* **1998**, *152*.

[2] A. Jagels, Y. Hövelmann, A. Zielinski, M. Esselen, J. Köhler, F. Hübner, H.-U. Humpf, *Mycotoxin research* **2018**.

## Compositional analysis of *lipid raft*-associated glycosphingolipids and phospholipids of human endothelial cells combining high-performance thin-layer chromatography and mass spectrometry

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**Introduction:** Glycosphingolipids (GSLs), composed of a hydrophilic oligosaccharide chain and a hydrophobic ceramide anchor, play pivotal roles in numerous biological processes including host-pathogen interaction. The ceramide moiety typically consists of the long-chain aminoalcohol sphingosine (d18:1), which is linked to a fatty acid with varying chain length (C16 to C24). GSLs localize primarily in phase-separated microdomains known as *lipid rafts*. GSLs and sphingomyelin (SM) are characteristic markers being embedded in the phospholipid matrix of *lipid rafts.* High-performance thin-layer chromatography (HPTLC) and mass spectrometry (MS) are invaluable tools for the separation of GSL mixtures and their structural characterization, respectively. However, neither HPTLC nor MS alone have the ability for precise structural determination of GSLs mostly due to the enormous heterogeneity of the oligosaccharide moiety built up from various sugar monomers (galactose, glucose, *N*-acetylglucosamine, *N*-acetylgalactosamine, etc.), their linkage type (1-3, 1-4, etc.) and anomeric configuration (α versus β). The employment of oligosaccharide-specific antibodies allows for detection of defined sugar epitopes of GSLs such as globotriaosylceramide (Gb3Cer) with Galα1-4Galβ1-4Glcβ1-1Cer or globotetraosylceramide (Gb4Cer) with GalNAcβ1-3Galα1-4Galβ1-4Glcβ1-1Cer structure. Combining the HPTLC overlay assay using GSL-specific antibodies and MS results in precise structural characterization of GSLs in complex mixtures.
**Objectives:** This study aimed at the structural clarification of Gb3Cer and Gb4Cer as well as their circumambient phospholipids in *lipid raft*-analogue detergent-resistant membranes (DRMs) prepared from primary human brain endothelial cells (pHBMECs) using the HPTLC overlay assay in conjunction with electrosprayionization (ESI) MS. Gb3Cer and Gb4Cer represent the canonical Shiga toxin (Stx) receptors expressed by pHBMECs, which are target cells of Stxs being released by human-pathogenic enterohemorrhagic *Escherichia coli* (EHEC) bacteria.
**Materials and Methods:** DRMs were prepared from pHBMECs by solubilization of the cells in Triton buffer. DRM and nonDRM fractions were obtained from discontinuous sucrose gradients upon ultracentrifugation. Gb3Cer and Gb4Cer were detected with specific polyclonal antibodies in the GSL preparations of the gradient fractions using HPTLC overlay immunostaining. The various Gb3Cer and Gb4Cer lipoforms as well as the phospholipids of the DRM and nonDRM fractions were structurally characterized using ESI MS.
**Results:** HPTLC overlay analysis of the neutral GSL fraction of pHBMECs gave separated double bands of Gb3Cer and Gb4Cer detected with the anti-Gb3Cer and anti-Gb4Cer antibody, respectively. MS analysis evidenced preponderance of Gb3Cer and Gb4Cer lipoforms harboring Cer (d18:1, C16:0), Cer (d18:1, C22:0) and Cer (d18:1, C24:0/C24:1) as lipid anchor. HPTLC immunostaining of the gradient fractions indicated prevalence of Gb3Cer and Gb4Cer in the DRM compared to the nonDRM fractions. Importantly, Gb3Cer and Gb4Cer species with saturated fatty acids dominated in the DRMs over the unsaturated counterparts in the nonDRMs. SM was detected in the DRM fractions only, whereas lyso-phosphatidylcholine (lyso-PC) was exclusively found in the nonDRM fractions, indicating association of SM with the liquid-ordered and lyso-PC with the liquid-disordered membrane phase.
**Conclusion:** We could show that the combination of HPTLC and MS analysis of GSL and phospholipid mixtures allows for precise compositional analysis of *lipid raft*-associated membrane lipids derived from human endothelial cells.