Short thesis for the degree of doctor of philosophy (Ph.D.)

COMPREHENSIVE *N*-GLYCOSYLATION ANALYSIS BY HIGH PERFORMANCE LIQUID SEPARATION TECHNIQUES

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INTRODUCTION

Most natural biopolymers such as oligo-nucleotide or -peptide molecules can be considered as flexible string-like structures due to the simple linear connection of their fundamental building blocks. Monosaccharides, on the other hand, can be combined to form up to thirty-two distinct disaccharides when all possible combinations of linkage position (connection at C-2, 3, 4, 6 or 8) and anomericity (α or β configuration) are considered. Common mammalian monosaccharides comprise glucose, mannose, galactose, *N*-acetylglucosamine (GlcNAc), fucose and sialic acid. Additionally, multiple residues can be attached to a single monosaccharide which can lead to complex branched structures. Therefore, oligosaccharides are amongst the most structurally diverse biopolymers in nature.

During or after transcription, newly assembled poly-peptide chains are frequently structurally altered by co- and/or post-translational modifications. The transformation of proteins to glycoproteins by the attachment of sugars to the protein backbone is a highly common modification. Sugar moieties, also commonly referred to as glycans, can be attached to asparagine (*N*-linked type glycans) with a conserved consensus sequence of Asn-Xxx-Ser/Thr (where Xxx is any amino acid but proline) or to threonine and serine (*O*-linked type glycans).

Glycosylation is a diverse but critically important post translational modification that modulates physical, chemical and biological properties of proteins. The

discovery of glycans' involvement in protein confirmation or function, cell-cell signaling and reflection of cellular or even organism physiological state has attracted major research attention. Alterations in glycosylation and the discovery of associated potential oligosaccharide-biomarkers were noted in a number of diseases including cancer.

Due to the analytical complexity associated with glycan analysis, a variety of analytical techniques is used, often in combined fashion, and generally including separation driven methods. Separation techniques such as HPLC and capillary electrophoresis (CE) hold an advantage over mass spectrometry based analyses due to their ability to separate isobaric oligosaccharide structures along with reliable quantitation. Particularly hydrophilic interaction liquid chromatography (HILIC) has consolidated its importance over the past 25 years due to robustness, reproducibility and the availability of databases that aid elution time based structural annotation. However, a limitation of HILIC-HPLC based analyses is the long analytical run time. New sub-2 μm HILIC phases for ultra performance liquid chromatography (UPLC) based glycomics facilitate increased efficiency but with significantly decreased analysis time. The ability to separate biologically significant linkage and conformational isomers at higher efficiency is of particular interest in the identification of disease associated glycosylation changes.

CE represents another rapid alternative analytical technique, offering differential electric field mediated separation selectivity. CE offers a variety of separation

modes, which incorporate different selectivity and associated glycan structure identification potential. The ability of separating both linkage and positional isomeric species based on inherent molecular shape differences, renders CE as a powerful tool for fine glycan structural analysis. However, robust and reproducible methods with associated extensive mapping databases that enable confident structural inference from normalized detection times of fluorescently derivatized glycans yet need to be introduced.

The application of multiple separation dimensions should be considered necessary to aid in elucidating the vast structural heterogeneity of glycans. Moreover, a parallel analysis route, introducing e.g. alternative labeling chemistry or orthogonal separation, can prove beneficial for method validation and preventing potential systematic bias. The combined application of complementary analytical approaches, such as HILIC and CE, shows promise for increased structural identification accuracy and confidence based on data integration from the individual dimensions.

The aims of this work were to:

- Transfer HILIC-HPLC *N*-glycan analysis methods to sub-2 μm HILIC stationary phase filled columns for UPLC to increase efficiency at decreased analysis time.
- Demonstrate the importance of separating glycan isomers of potentially associated biological significance at higher efficiency and underline the applicability of the method in identifying disease induced glycosylation changes.

- Develop a CE method including eligible normalization approaches to ensure experimental precision as well as structural elucidation accuracy, matching those of the established HILIC/UPLC approaches.
- Highlight the applicability of the method and the exceptional resolution of CE by the investigation of disease related *N*-glycan alterations.

- Compare the two analytical methods and highlight their inherent merits and shortcomings
- Investigate the potential of a combined parallel application to further decrease experimental expenditure but in the same time increase glycan structural elucidation accuracy and confidence.

MATERIALS AND METHODS

Serum samples and small scale 2-DE for stomach cancer study.

Blood samples were collected from 80 patients with stomach cancer, (18 Level I, 26 Level II, 23 Level III and 13 Level IV), 10 with benign stomach disease and 20 matched controls. To minimize biological variance, 5 µL of serum from each patient was pooled according to their pathological classification to yield disease specific patient pools.

A volume of pooled serum corresponding to 150 µg of total protein was loaded onto pH 4-7 strips for isoelectric focusing. Focused strips were reduced, alkylated and subjected to second dimension SDS-PAGE separation. Coomassie stained protein spot features were excised.

Extraction of IgG from pooled control serum.

Immunoglobulin G (IgG) was extracted using a Protein A column from healthy human serum (n=40, median age=29, 11 males, 29 females). After elution at low pH, IgG was buffer exchanged to neutral pH. Protein purity was checked with 10% reducing SDS-PAGE.

Haptoglobin purification for pulmonary disease study

Age and gender matched human control plasma (2 males, avg. age 61.0), pneumonia plasma (3 males, avg. age 60.3), COPD plasma (3 males, avg. age 61.6) and lung cancer plasma (3 males, average age 61.3) were collected with informed consent. After

albumin and IgG depletion, haptoglobin was partitioned from the plasma samples of patients using specific monoclonal antibodies. Protein purity was checked by SDS-PAGE.

N-glycan release, labeling and post-derivatization clean-up

Sugar moieties were enzymatically liberated from intact (IgG) or reduced and alkylated glycoproteins (human serum, β-glucuronidase, haptoglobin) *via* in solution PNGaseF digestion, the enzyme was used at 10% of the final digest volume. For the stomach cancer study, excised 2-DE gel spots were processed by in-gel PNGaseF digestion. Glycans were fluorescently derivatized *via* reductive amination with 2-aminobenzamide (2-AB) for UPLC analysis or 8-aminopyrene-1,3,6-trisulphonic acid (APTS) for CE-LIF analysis. Excess fluorophore was removed by HILIC phase packed pipette tips. Structural annotation of the chromatographic peaks was performed by comparison of retention time data expressed as glucose units (GU) values with the GlycoBase database (NIBRT, Ireland).

Enzymatic N-glycan processing and affinity purification

All exoglycosidase digestions were performed in ammonium acetate, pH 5.5, at 37°C overnight. Phosphorylated oligosaccharides or phosphorylated glycopeptides were selectively enriched using TiO_2/ZrO_2 mixed oxide affinity purification. Alkaline phosphatase digests were carried out in Tris-HCl, pH 9.3, at 37°C overnight.

HILIC UPLC-fluorescence glycan profiling

2-AB labeled *N*-glycans were separated by UPLC with fluorescence detection on a Waters Acquity instrument. Separations were performed using Waters BEH Glycan column, 100 x 2.1 mm i.d., 1.7 μm BEH particles, using a linear gradient of 70-53 % acetonitrile at 0.56 ml/min in 16.5 minutes. Retention times were converted into glucose unit (GU) values by time based standardization against a dextran hydrosylate ladder.

Anion exchange chromatography based fractionation

Separation of the *N*-glycan pool based upon the degree of sialylation present was performed by anion exchange chromatography (AEC). A linear gradient of 100 mM acetate, pH 7.0 in 20% v/v acetonitrile was used. Samples for CE-LIF analysis were injected in their unlabeled form and each of the collected fractions was subsequently labeled with APTS.

CE-LIF N-glycan profiling

CE separations of APTS labeled *N*-glycans were performed on a Beckman PA800 Plus in an N-CHO neutral coated capillary (50 μm I.D; 60 cm total, 50 cm effective length) using laser induced fluorescence detection (LIF). For all experiments a commercially available carbohydrate separation buffer was used (Beckman Coulter) at a separation temperature of 25°C and using an applied potential of -30 kV (500 Vcm^{-1}). To improve precision, two bracketing standards were used. Migration times were converted to relative migration times using the

bracketing standard boundaries. Normalized migration times were converted to GU values using a time based standardization against a malto-oligosaccharide ladder.

Protein ID with LC-MS/MS for stomach cancer study

Deglycosylated 2-DE gel spots were digested overnight with trypsin and peptides eluted from the gel pieces. LC-MS/MS analysis was performed using a nano scale HPLC instrument connected *via* chip interface to an ion trap mass spectrometer operated in positive ion mode with a spray voltage of -1.8kV. Separations were performed using an enrichment column and a 43 mm analytical column packed with 300 Å 5 μm C18 particles. Peptides were eluted using a linear gradient of 5-60 % acetonitrile containing 0.1 % v/v formic acid. Data dependent MS/MS data was searched against the NCBI database using the human mouse taxonomic filter.

N-Glycosylation site analysis of β-glucuronidase

Protein aliquots were reduced, alkylated and digested with either Asp-N or Lys-C overnight at 37°C. Resulting peptides were separated, collected and processed to characterize the *N*-glycosylation site to which oligosaccharides displaying mannose-6-phosphate were attached.

Peptide mapping was performed using a 3.5 μm, 135 Å C_{18} 150 x 2.1 mm i.d. column with UV/Visible detector. Peptides were eluted using a linear gradient of 5-65% acetonitrile containing 0.1% v/v TFA in 55 minutes at a flow rate of

200 µL/min. Chromatographic peaks were individually collected, deglycosylated and subjected to glycosylation profiling by UPLC and peptide identification using LC-MS/MS.

LC-MS/MS analysis of enriched deglycosylated peptides was performed using a nano LC system connected to a LTQ Orbitrap XL mass spectrometer. Separations were performed using an increasing linear gradient of acetonitrile on a 0.075 x 150 mm C_{18} column at 300 nL/min. The mass spectrometer was operated in positive ion mode, the spray voltage was -1.8kV. MS/MS data were searched against the Uniprot-Swissprot database with the Homo sapiens taxonomic filter specified.

Statistical analysis

Non parametric Kruskall-Wallis analysis of all glycomic data was used to evaluate differences in the median values across pathological groups. Differences between variables were further investigated using with Mann-Whitney or Students t-tests. In all instances, p values < 0.05 were considered as being statistically significant.

RESULTS AND DISCUSSION

UPLC Profiling of Serum N-glycans for Fast and Efficient Identification of Cancer Associated Alterations

Compared to HILIC-HPLC, significant improvements in efficiency, selectivity and analysis speed offered by UPLC profiling of fluorescently labeled *N*-linked oligosaccharides on a recently introduced sub-2 μm HILIC stationary phase enabled the identification of stomach adenocarcinoma associated alterations. The increase in chromatographic efficiency permitted the separation of positional and linkage specific isomeric oligosaccharides and also glycans containing a bisecting GlcNAc residue. The ability to resolve and reliably determine lower abundant and biologically significant oligosaccharide species represents a considerable advance in glycomics separations. This is particularly relevant when searching for cancer associated alteration in glycosylation or attempting to gain an understanding into the physiology of the disease. In total, 35 out of the 45 serum *N*-glycan chromatographic peaks were returned as being statistically altered *versus* pathological classification. Lower levels of asialo- and monosialylated core fucosylated glycans, normally carried on IgG molecules, and an increase in branching and sialylation of *N*-glycans was observed, that represents a commonly reported hallmark of cancer. The contribution of the glycosylation present on four highly abundant proteins, IgG, haptoglobin, transferrin and α1-acid glycoprotein was evaluated. Their individual glycans appeared to populate

the majority of the serum glycome, however, the presence of the same chromatographic peaks after immunoaffinity depletion indicated the presence of these biologically significant glycans also on other lower abundant serum proteins. Alterations in the glycosylation present on these four proteins isolated from the pathologically staged cancer serum using either affinity purification or two-dimensional electrophoresis (2-DE) were then investigated as possible markers for stomach cancer progression. Protein identity of the excised 2-DE gel spots was confirmed by LC-MS/MS. In agreement with previous reports an increase in sialylation was observed on haptoglobin, transferrin and α1-acid glycoprotein in the cancerous state. Increased levels of agalactosyl *N*-glycans on IgG with disease progression can be associated with previously reported pro-inflammatory properties of IgG isoforms carrying agalactosyl *N*-glycans which, when clustered are a ligand for mannose binding lectin and complement activation.

High-efficiency and robust CE analysis reflects molecular conformation and configuration variation of isomeric oligosaccharides

A practical method for rapid carbohydrate profiling by CE including eligible normalization approaches, that ensure experimental precision and comparability of delicate migration differences between structurally similar or isomeric species, is presented. Fluorescently derivatized isomeric malto-, cello- and isomaltooligosaccharide ladders, differing only in their linkage type of α1-4, β1-4 and α1-6, respectively, as well as a sterically larger *N*-acetylchito-oligosaccharide ladder were used as

model compounds. The introduction of a lower and an upper internal bracketing standard enabled the precise alignment of the different homooligosaccharide CE profiles, thus their fine comparison. Mere differences in glycosidic linkage type or anomericity of isomeric oligosaccharides revealed differential migration, thus reflecting the separation principle based on discrepancies in hydrodynamic radii and associated molecular conformations. Migration differences between respective α- and β-linked oligosaccharides were more pronounced, whereas isomeric α1-4 and α1-6 linked species exhibited almost identical migration behavior in the lower degree of polymerization range of DP 2-6, but diverged commencing with DP 7. Fundamental electrophoretic mobility theory was used to further investigate the correlation between changes in hydrodynamic volume and corresponding electrophoretic characteristics of oligosaccharides with different molecular properties. In agreement with molecular modeling and previous reports, the presented results reflect that the more flexible equatorially oriented bond in the β-anomeric configuration leads to extended rod-like structures, whereas the axial α1-4 linkage is bent intending to form helical structures supported by intramolecular H-bonds. With increasing size, these H-bonds stabilize the helical conformation of maltooligosaccharides, thus rendering α1-4 linked oligo-glucoses to form a more compact and less flexible helical structure than that of its linear β1-4 linked counterpart. Based upon the tendency of α1-4 linked maltooligosaccharides to form a full helical turn upon reaching DP 6 and the fluorescent labeling induced reducing terminal open ring

form, differences to respective β1-4 homooligomers consolidated from DP>7. Additionally, the impact of hydrogen bridges, and associated availability of hydroxyl groups, on the molecular conformations were confirmed by HILIC. Due to the different separation mechanism in HILIC; however, geometrical changes towards the completion of a full helical turn in maltohexaose or -heptaose were not as apparent as in CE studies.

The high precision of the presented method, induced by internal alignment standards and the suggested migration time based normalization against a maltooligosaccharide ladder (i.e. replace migration time by glucose units) make it ideal for comparative studies of biomedical interest by CE.

Aptitude of CE to reveal pulmonary disease associated glycosylation features and alterations of haptoglobin N-glycans

The developed robust CE based method was applied to compare the *N*-glycosylation profile of haptoglobin in normal and pathologic conditions. Haptoglobin was isolated from plasma samples of a small scale cohort of healthy, pneumonia, chronic obstructive pulmonary disease (COPD) and lung cancer patients by two haptoglobin specific monoclonal antibodies. *N*-glycan structure elucidation of a haptoglobin protein standard was achieved by targeted exoglycosidase digestions and sophisticated features, such as core or antennary fucosylation present on isomeric species were successfully accentuated by CE-LIF. The structures were then recognized in the clinical samples by horizontal alignment based on the internal

standards and comparison of the corresponding glucose unit values. Potential disease associated changes in the levels of individual glycan species were investigated in the different patient groups. The comparison of control versus COPD revealed a significant decrease in the amount of bi- and an increase in tri-antennary glycans, also indicating elevated branching in this group. Additionally, α1-6 fucosylated tri-antennary glycans were increased in all disease groups and elevated core- and arm fucosylation on tetra-antennary glycans were detected in the lung cancer compared to the COPD group.

The current study, however, rather represents a proof of concept approach wherein the potential of CE-LIF for the robust profiling of complex glycans pools including positional and/or linkage isomers (e.g. core or antennary fucosylation) of biomedical interest is demonstrated. Gender and age variability of the *N*-glycans present on serum glycoproteins require large scale studies in order to claim biomarker or diagnostic conclusions.

From comparison to unity of HILIC-UPLC vs. CE-LIF: Rapid and Confident IgG N-glycan Structural Elucidation

UPLC and CE-LIF both revealed excellent but differential selectivity for *N*-glycans released from healthy human serum polyclonal IgG. Using the applied buffer system, CE-LIF was outperformed by the 1.7 μm HILIC phase for the separation of neutral glycans and their afucosyl and bisecting GlcNAc containing analogues. CE-LIF on the other hand displayed superior selectivity for the separation of sialylated

structures, in particular, monosialylated glycans wherein it was possible to separate fucosylated and afucosylated structural analogues in a region of the electropherogram prior to the migration of all neutral structures. Both HILIC and CE-LIF required very similar cycle times (injection-to-injection). A further advantageous feature of CE-LIF was the significantly lower sample consumption per injection, as CE injection volume is on average three orders of magnitude lower than that onto the UPLC column.

Structure annotation of all 32 potential structures in the human IgG *N*-glycan pool was achieved by a combination of weak anion exchange chromatography (AEC) fractionation and exoglycosidase digestion steps with subsequent profiling and necessitated over 48 hours. Upon combination of the data sets generated individually by UPLC and CE-LIF using a 2D approach, all 32 oligosaccharides present are well separated within the confined two-dimensional space. The use of glucose unit values following time based normalization and the implementation of CE-LIF bracketing standards further increase injection-to-injection reproducibility by eliminating any associated experimental variance. Combining this high precision with the beneficial orthogonality of the data sets allowed for exhaustive and confident structural annotation of the human IgG *N*-glycan pool within only 20 minutes using the 2D space. Simultaneous use of both analytical techniques offers a powerful platform for rapid and comprehensive analysis of IgG *N*-glycosylation present on therapeutic antibodies or on antibodies of biomedical or pathological significance.

Identification of N-Glycans Displaying Mannose-6-Phosphate and their Site of Attachment on recombinant β-glucuronidase

The acid hydrolase β-glucuronidase is a 74.7 kDa lysosomal enzyme, which exists as a homotetramer, exhibiting four *N*-glycan sites on each monomer at asparagines 173, 272, 420 and 631. The addition of mannose-6-phosphate (M6P) to *N*-glycans on acid hydrolases is a critical process in the targeting of enzymes to the lysosome and malfunction of this system results in several pathological conditions. Characterization of mono- and bis-M6P bearing oligosaccharides poses a considerable analytical challenge. HILIC-UPLC profiling and CE-LIF combined with exoglycosidase digestion and weak anion exchange fractionation for the characterization of M6P bearing glycans on recombinant β-glucuronidase expressed in Chinese Hamster Ovary (CHO) cells, was investigated. Using this multidimensional approach a number of peaks were observed to resist exoglycosidase digestion, suggesting the presence and blocking activity of the M6P tag. AEC fractionation indicated the presence of a charged substituent other than sialic acid. To investigate further, mixed metal oxide affinity purification on a combined TiO_2/ZrO_2 resin facilitated the selective enrichment of oligosaccharides bearing mono- or diphospho esters that corresponded to those peaks previously identified to resist exoglycosidase digestion. Alkaline phosphatase digestion identified $Man_6GlcNAc_2$ and $Man_7GlcNAc_2$ glycans as the primary carriers of the M6P tag. Reconciliation of the HILIC-UPLC data for phosphorylated Man_7GlcNA_2-M6P was more complicated

due to the potential presence of positional α1-2 linked mannose isomers and additionally the location of the phosphate residue on those terminal mannose residues. A total of eight $Man_7GlcNAc_2$-M6P possible monoesters led to the multiplicity in the resulting chromatogram. The presence of the phosphate residue also results in peak broadening potentially due to secondary anion exchange interactions with the amide-functionalized HILIC phase. Orthogonal separation in CE-LIF, wherein the addition of phosphate residues and associated negative charges resulted in distant earlier migration, proved to be an important factor in identifying M6P on both $Man_6GlcNAc_2$ and $Man_7GlcNAc_2$. The ability to cross correlate the data individually generated using both techniques increased the confidence of the oligosaccharide structural assignments.

In agreement with previous studies, site-specific glycoproteomic analysis revealed that $Man_7GlcNAc_2$-M6P oligosaccharides were present at asparagine 272 and 420, while asparagine 631 displayed $Man_6GlcNAc_2$-M6P. The oligosaccharides carrying the M6P residue were not structurally characterized before. The analytical strategy applied herein represents a novel yet simple approach for the qualitative and semi-quantitative structural characterization of M6P containing oligosaccharides on therapeutic enzymes.

CONCLUSIONS

Compared to HILIC-HPLC, significant improvements in efficiency, selectivity and analysis speed offered by the sub-2 μm HILIC phase make it an ideal tool for rapid yet highly efficient separation of complex oligosaccharide mixtures such as that present in the serum *N*-glycome. The presented novel application of HILIC-UPLC has already established as the basis for further serum glycomic biomarker discovery experiments.

The developed CE based method also enabled rapid and high efficiency separations of complex carbohydrate pools. Mere differences in linkage type or anomericity of isomeric oligosaccharides revealed differential migration, thus reflecting the separation principle based on discrepancies in hydrodynamic radii and associated molecular conformations. High precision induced by internal standards and GU normalization permits comparative studies of biomedical interest by capillary electrophoresis.

In a proof of concept study, such complex *N*-glycan features as core or antennary fucosylation, altered in inflammatory and malignant lung diseases, were successfully accentuated by CE-LIF. The standardized CE method not only revealed its potential for large scale studies, but can also pave the way for a database enabling direct deduction of carbohydrate structural information from experimental CE data, similar to the established HILIC-HPLC sources.

The combined use of both UPLC and CE-LIF is recommended for rapid and comprehensive characterization of glycosylation present on antibodies for therapeutic administration or in the biomedical setting in order to maximize the analytical return and permit translation of the resulting data into a meaningful biological interpretation. The unified analytical platform opens the possibility for vector based annotation of unknowns based upon their positioning within the multidimensional data cloud and furthermore allows for the statistical analysis of the vector based positional match. This expands the potential for a 'score' based confidence system in the resulting annotation as routinely used in other 'omics' data analysis.

The orthogonal selectivity of UPLC and CE decisively contributed to the comprehensive characterization of mono- and *bis*-mannose-6-phosphate bearing oligosaccharides present on recombinant β-glucuronidase. Considering the criticality of M6P determination to ensure enzyme efficacy, the presented approach represents a significant analytical advance for the structural characterization of recombinant therapeutic enzymes used for lysosomal storage disorders treatment allowing for the determination of M6P at both the oligosaccharide and glycopeptide levels.

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APPENDIX I

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Doctoral School: Doctoral School of Molecular Medicine

List of publications related to the dissertation

1. **Mittermayr, S.**, Bones, J., Guttman, A.: Unraveling the Glyco-Puzzle:Glycan Structure Identification by Capillary Electrophoresis.
   Anal. Chem. Epub ahead of Print (2013)
   DOI: http://dx.doi.org/10.1021/ac4006099
   IF:5.856 (2011)

2. Váradi, C., **Mittermayr, S.**, Szekrényes, Á., Kádas, J., Takács, L., Kurucz, I., Guttman, A.: Analysis of Haptoglobin N-glycome Alterations in Inflammatory and Malignant Lung Diseases by Capillary Electrophoresis.
   Electrophoresis. Epub ahead of Print (2013)
   DOI: http://dx.doi.org/10.1002/elps.201300041
   IF:3.303 (2011)

3. **Mittermayr, S.**, Guttman, A.: Influence of molecular configuration and conformation on the electromigration of oligosaccharides in narrow bore capillaries.
   Electrophoresis. 33 (6), 1000-1007, 2012.
   DOI: http://dx.doi.org/10.1002/elps.201100681
   IF:3.303 (2011)

4. Bones, J., **Mittermayr, S.**, McLoughlin, N., Hilliard, M., Wynne, K., Johnson, G.R., Grubb, J.H., Sly, W.S., Rudd, P.M.: Identification of N-Glycans Displaying Mannose-6-Phosphate and their Site of Attachment on Therapeutic Enzymes for Lysosomal Storage Disorder Treatment.
   Anal. Chem. 83 (13), 5344-5352, 2011.
   DOI: http://dx.doi.org/10.1021/ac2007784
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5. **Mittermayr, S.**, Bones, J., Doherty, M., Guttman, A., Rudd, P.M.: Multiplexed Analytical Glycomics:Rapid and Confident IgG N-Glycan Structural Elucidation.
J. Proteome Res. 10 (8), 3820-3829, 2011.
DOI: http://dx.doi.org/10.1021/pr200371s
IF:5.113

6. Bones, J., **Mittermayr, S.**, O'Donoghue, N., Guttman, A., Rudd, P.M.: Ultra Performance Liquid Chromatographic Profiling of Serum N-Glycans for Fast and Efficient Identification of Cancer Associated Alterations in Glycosylation.
Anal. Chem. 82 (24), 10208-10215, 2010.
DOI: http://dx.doi.org/10.1021/ac102860w
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The Candidate's publication data submitted to the Publication Database of the University of Debrecen have been validated by Kenezy Life Sciences Library on the basis of Web of Science, Scopus and Journal Citation Report (Impact Factor) databases.

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