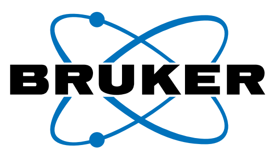

Application Note # LCMS-84

Combining PGC-LC ESI and MS²/MS³ ion trap analysis for structural characterization of sulphated *O*-glycans

Introduction

Human mucosal surfaces contain high concentrations of heavily *O*-glycosylated mucin glycoproteins that form a protective coating. This has been recognised as an important key player of the human innate immune system [1] and defects in mucin glycosylation have also been linked with various diseases such as cystic fibrosis, chronic obstructive pulmonary disease (COPD) or asthma [2]. Since these mucin *O*-glycans present a large variety of very different glycan epitopes on their surface, selective and automatable screening methods with the capacity for in-depth structural characterization represent key technologies for studying disease related *O*-glycosylation pattern. In contrast to *N*-glycosylation, *O*-glycans are usually found to contain more structural varieties due to the presence of several core structures [3]. This, however, poses particular analytical challenges in structure determination since less information can be inferred in structure interpretation compared to the analysis of *N*-glycans.

Porous graphitized carbon (PGC) LC-ESI ion trap MS^n analysis provides an attractive alternative to elucidate fine structural details that otherwise are difficult if not impossible to differentiate from the limited amount of

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biological sample material [4,5]. Using human saliva as an easily available source of mucin type *O*-glycans, the advantages of ion trap MS^n in combination with PGC separation for detailed structure determination is exemplified on a sulphated tetrasaccharide *O*-glycan detected in four different isoforms.

Experimental

Sample preparation

Saliva (2 mL) was collected from healthy individuals and centrifuged at 3000 x g and 4°C for 20 min to precipitate cells [5]. 1 ml of the supernatant was vacuum dried. Glycans were chemically released by β-elimination adding 200 µL 0.5 M $NaBH_4$ in 50 mM KOH and further incubation for 16 h at 50 °C before stopping the reaction by adding 20 µL acetic acid. After β-elimination glycans were desalted and purified with PGC-SPE as described by Jensen et al. [6] and vacuum dried.

LC separation

Porous Graphitized Carbon Liquid Chromatography (PGC-LC) was performed on an Ultimate 3000 UHPLC system (Thermo Fisher) that was online coupled to an amaZon speed ETD ion trap mass spectrometer (Bruker Daltonics). Dried samples (see above) were dissolved in 20 µL water and 3 µL were separated by PGC-LC (Hypercarb, 30 x 0.32 mm, 5 µm (pre-column), 100 mm x 0.18 mm, 3 µm (separation column, Thermo Hypersil).

The sample was loaded on the pre-column at a flow rate of 6 µL/min in buffer A (10 mM ammoniumbicarbonate) for 5 min before a gradient of 15.8% to 40.3% buffer B (60% ACN in 10 mM ammoniumbicarbonate) over 49 min at a flow-rate of 1 µL/min was applied. The LC system was connected to the mass spectrometer using the nano-flow ESI sprayer (Bruker Daltonics).

Mass spectrometric analysis

The MS settings were as follows: capillary outlet 4500 V, end plate voltage 500 V. Nebulizer was set to 5 psi and desolvation gas temperature was 220°C at a flow-rate of 5 L/min.

Negative ion mode MS spectra were obtained within a mass range of 380 - 1800 *m/z*. Smart parameter setting (SPS) was set to 900 *m/z*. For MS/MS precursor selection the three most intense ions were isolated with a width of 1.5 *m/z* (including singly charged ions) above the absolute intensity of 34.000 and 20% relative intensity threshold. The ion charge control (ICC) was set to 40.000 with a maximum accumulation time of 200 ms. CID was performed using helium as collision gas. The fragmentation amplitude was set to 100% using SmartFrag™ Enhanced for amplitude ramping (30 - 120%). Fragmentation time was set to 32 ms.

MS^3 analysis

For negative ion mode MS^3 analysis the multiple reaction monitoring (MRM) feature of the trapControl 7.1 acquisition software was used. The MS^2 precursor 829.3 *m/z* was isolated with an isolation width of 4 m/z and fragmented with an amplitude of 120%. This fragmentation generated the MS^3 precursor 444.2 *m/z*, which was isolated with a width of 3 m/z and fragmented with an amplitude of 180%. SPS was set to 800 *m/z*.

Data processing and glycan identification

MS data were processed using DataAnalysis 4.1. Mass annotation settings were adapted to the intensities of the MS/MS signals. Deconvoluted MS/MS signals were exported as singly charged masses.

Glycan MS^2 spectra were imported in ProteinScape 3.1, where the negative charge of the signals was detected automatically. The glycan database search was performed against Glycome DB (www.glycome-db.org) using the search engine GlycoQuest, which is integrated in ProteinScape. Search parameters used are listed in Table 1.

Table 1: GlycoQuest search parameters

Parameter	Value
Submitted to search	All spectra
Glycan type	-
Taxonomy	No restriction
Database	GlycomeDB
Composition restriction	Fuc < 4; Hex < 9; HexNAc < 6; NeuAc < 3
Modifications	Sulphate (0-2)
Derivatization	Underivatized
Reducing end	Reduced end
Ions	H^+ up to 1, charge permutation in negative mode 1 to 3
MS tolerance	0.4 Da
MS/MS tolerance	0.5 Da
$\#^{13}C$	1
Fragmentation	A, B, C, X, Y, Z; max. 3 cleavages; max. 1 cross-ring cleavage
Threshold: Score	> 2
Threshold: Fragmentation coverage [%]	>10
Threshold: Intensity coverage [%]	>10

Results

Stage 1: MS and MS^2 composition analysis

Reductive β-elimination represents an simple way for releasing *O*-glycans from glycoproteins for subsequent structural characterization. PGC-LC provides an easy opportunity for separation of unlabelled, isobaric glycan components that are essentially impossible to differentiate by simple mass spectrometric approaches [3,4,6]. The base peak chromatogram (BPC) separation profile determined for a complex mixture of saliva *O*-glycans (Figure 1, top) exemplifies the separation of just some *O*-glycan structures successfully identified by and the search engine GlycoQuest.

In recent years, in-depth structural glycan characterization, e.g. localization of additional modifications or linkage elucidation, has been done by performing experiments with specific exoglycosidases and several LC-analyses. However, ESI ion trap MS^n in combination with PGC-LC provides a more straight-forward approach for glycan characterization. Two subsequent analyses are sufficient to yield comprehensive and specific information on the glycan structures of interest. Importantly, PGC-LC ESI MS^n is also fully compatible with traditional exoglycosidase digestion protocols providing a further level of structure verification.

In the example presented here, a simple tetrasaccharide with the composition $Hex_2HexNAc_2Sulph$ ($[M-H]^-$ = 829.24 Da) was found to be present in four distinct isoforms eluting at 20.1, 21.6, 23.5 and 25.7 min, respectively (Figure 1, bottom).

The sum spectrum in Figure 2 (bottom), shows the MS^2 spectrum obtained without any prior separation. The differentiation and assignment of individual *O*-glycan isoforms is nearly impossible since the observed fragments derive from different isoforms with the very same *m/z*-value. In contrast, the signature MS^2 spectra of the four isobaric sulphated tetrasaccharides (Figure 2, spectra 1-4) provide specific structural information as deduced from several diagnostic ions (see also Table 2) since they were separated by PGC-LC into distinct peaks. However, a considerable degree of uncertainty remained. Especially, the position of the sulphate group and more detailed linkage information could not unambiguously be determined in this step for all structures.

In the case of structure 2 (RT 21.6 min) a very distinct MS^2 spectrum already indicated the sulphate group to be attached to the core-galactose (464.22 *m/z*, Figure 2 and Table 2). For more details on specific diagnostic ions please refer to [4]. A prominent Hex-HexNAc-sulphate-fragment (444.2 *m/z*) was found (1,3,4) for the three remaining isoforms (Figure 2). This fragment, however, just indicated the sulphate to be present on one of the outer arms of the respective glycan structures. Less intense fragments already provided a good indication regarding which monosaccharide residue the sulphate is attached to. Nevertheless, clear evidence and supportive linkage information was not obtained, and considering the fact that a sulphate migration can occur to some extend during CID fragmentation [7], further MS^3 experiments were performed.

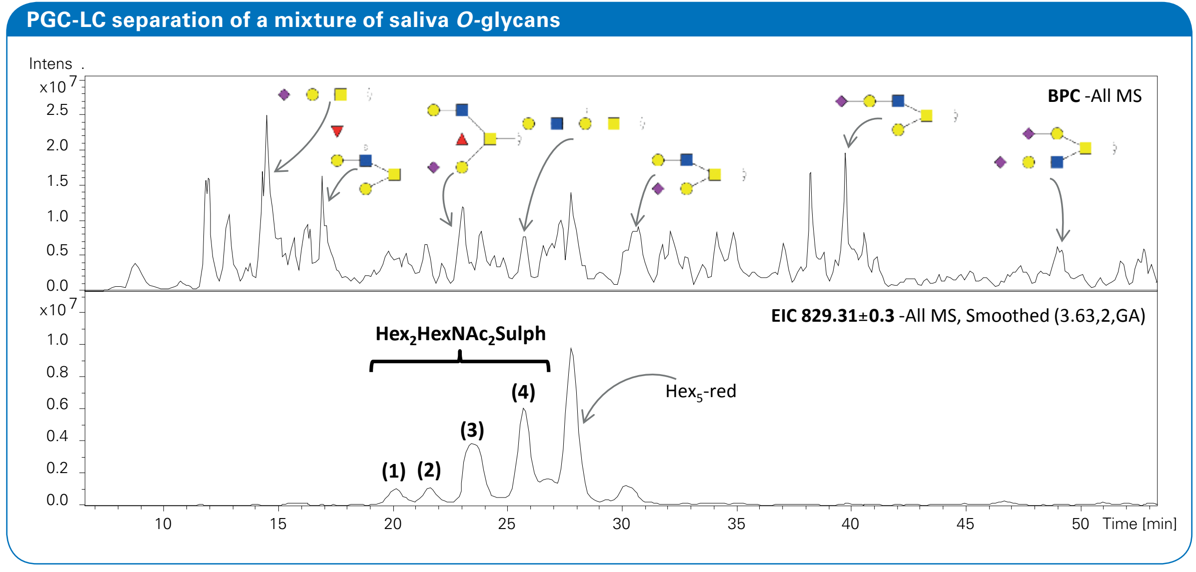

Figure 1: Top: Base peak chromatogram illustrating the complexity and variety of mucin *O*-glycans. Bottom: Extracted ion chromatogram of the 829.31 *m/z* representing four different isoforms of the sulphated tetrasaccharide ($Hex_2HexNAc_2Sulph$; Cmpd. 1,2,3,4) and a reduced Hex5.

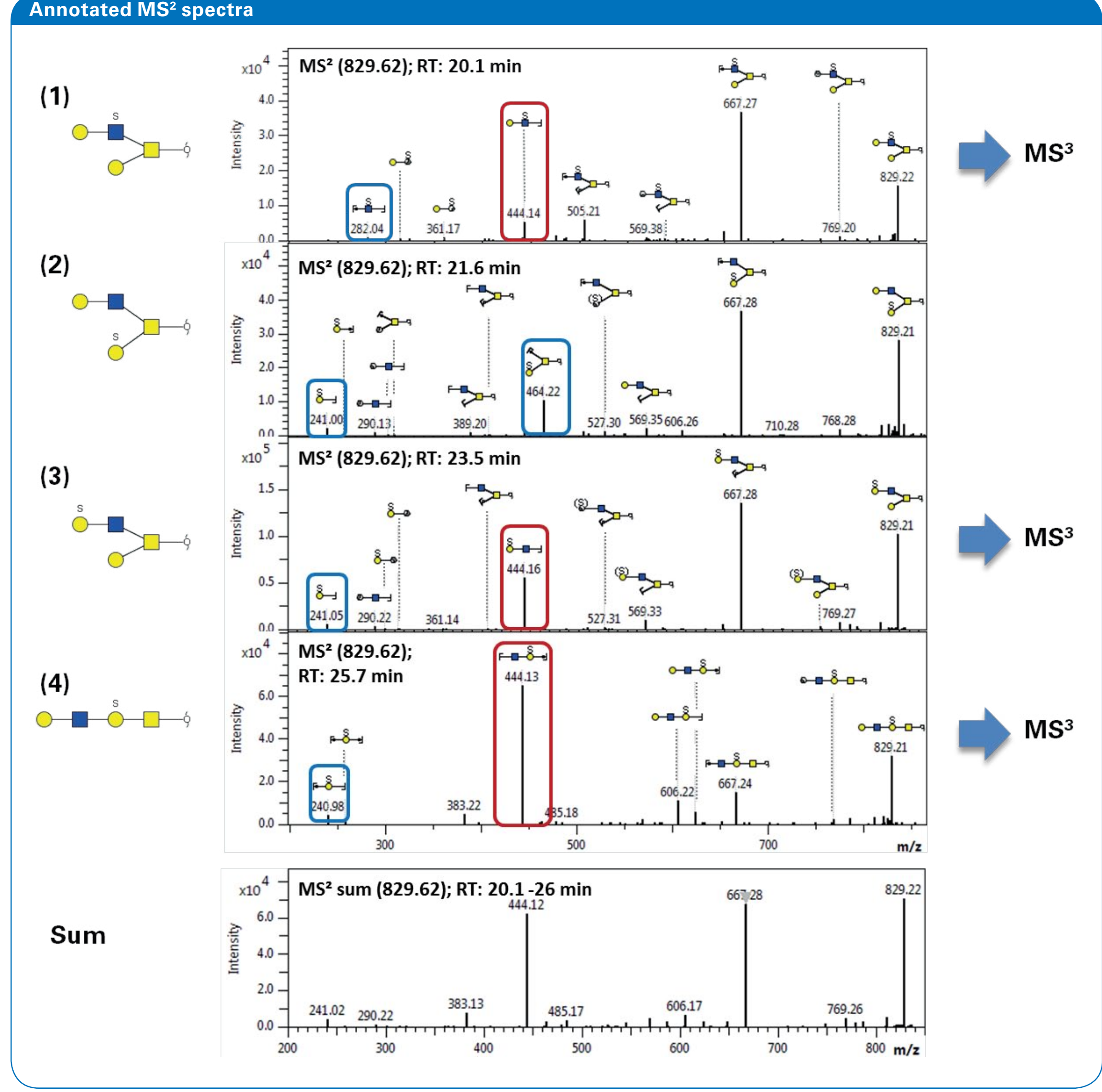

Figure 2: Annotated MS² spectra (identified with GlycoQuest) of the PGC-LC separated (1-4) and non-separated (Sum) sulphated tetrasaccharide ($Hex_2HexNAc_2Sulph$) with the singly charged precursor 829.62 **m/z**. Diagnostic fragment ions illustrated in Table 2 are marked with a box. Compounds 1, 2, and 4 with the fragment ion 444.1 *m/z* were further evaluated in MS³ experiments.

Stage 2: Targeted MS³ structure determination

Using the same analytical equipment, a targeted MS³ experiment was designed based on the MS² results. The MRM option of the amaZon speed control software [5] allowed to specifically select the singly negatively charged signal 444.1 *m/z* (sulphated HexHexNAc fragment) derived from the singly negatively charged precursor 829.24 *m/z* for targeted MS³ experiments (MS³: 829.24->444.10). With the resulting MS³ spectra it was possible to clarify individual structural features such as sulphate localization (HexNAc-Sulph or Hex-Sulph, Figure 3). Specific cross ring cleavages were also helpful in the assignment of glycosidic linkages.

Table 2: Diagnostic fragments observed in MS² spectra (negative ionization mode)

Fragment composition	$[M-H]^-$ (calculated)
Hex-Sulph	241.00, 259.01
HexNAc-Sulph	282.03, 300.04
Hex-HexNAc-Sulph	444.08
Hex-HexNAc(red)-Sulph	464.11

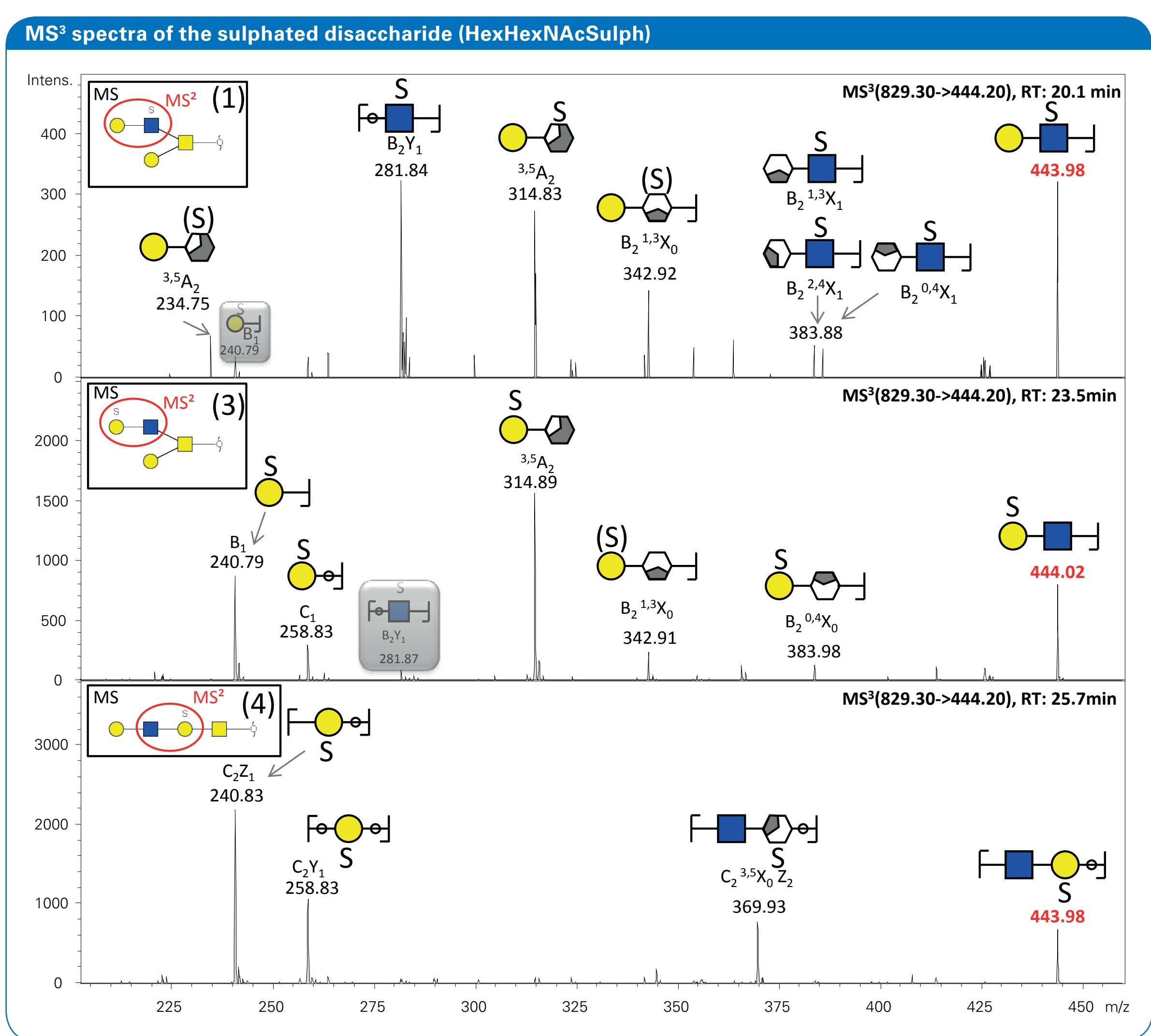

Figure 3: MS³ spectra of the sulphated disaccharide (HexHexNAcSulph) with the singly charged precursor 444.1 *m/z* generated during MS² fragmentation of compounds 1, 3 and 4.

In case of compound 1 (RT 20.1 min) the intense B_2Y_1 fragment (281.84 *m/z* clearly allows the assignment of the sulphate group to the core 2 GlcNAc. Furthermore, the crossring cleavage fragments $^{3,5}A_2$ (314.83 *m/z*) and B_2 $^{1,3}X_0$ (342.92 *m/z*) suggest a 1,4 linkage of the hexose to the GlcNAc and the sulphate being attached to C6 position of the core 2 GlcNAc. This assignment was made based upon the current knowledge on *O*-glycan biosynthesis where the hexose is only described as being attached via 1-3 and 1-4 linkages to the core 2 GlcNAc (Figure 3, top spectrum). The B_1 ion, (240.79 *m/z*), indicating a sulphated hexose, results from traces of sulphate migration during the CID process. This further supports the assignment described above since localization of the sulphate on a C6 has been reported to enhance the migration during the CID process [7].

In a similar manner, the MS³ spectra of compound 3 (RT 23.5 min) clearly indicated the sulphate to be attached to the hexose (intense B_1 ion, 240.79 *m/z*). Based on the $^{3,5}A_2$ (314.89 *m/z*) and B_2 $^{1,3}X_0$ (342.92 *m/z*) ions the hexose was again interpreted to be linked via the C4 to the core 2 GlcNAc residue (Figure 3 middle spectrum). The phenomenon of sulphate migration here also explains why traces of a B_2Y_1 ion (281.87 *m/z*) were detected.

In the case of compound 4 (RT 25.7 min) the localization of the sulphate group on the hexose can be again explained by the abundant fragment ion C_2Z_1 (240.83 *m/z*). Here, no signals indicating a sulphate migration were detected. Furthermore, the MS³ spectrum and cross-ring fragment C_2 $^{3,5}X_0$ Z_2 (369.93 *m/z*) indicated that the HexNAc and the sulphate group were attached on the C3 and C2 of the galactose, though these results do not allow to differentiate whether the HexNAc or the sulphate group are attached to the C2 or C3, respectively (Figure 3 bottom spectrum).

An overview over the received results with more detailed glycan structure assignment is shown in Figure 4.

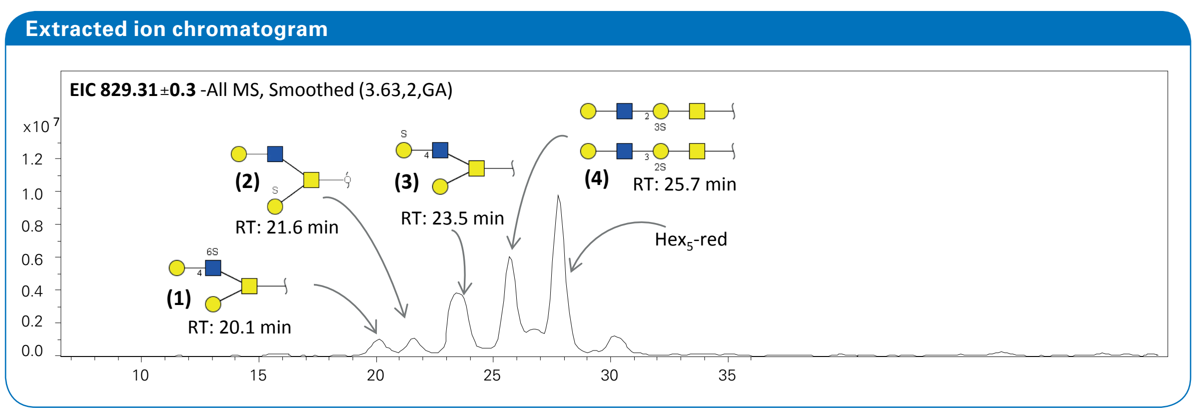

Figure 4: Extracted ion chromatogram (829.31 *m/z*) of the sulphated tetrasaccharide ($Hex_2HexNAc_2Sulph$) showing the structural assignment of these mucin *O*-glycans. Sulphate and linkage annotation is deduced from their specific MS^2 and MS^3 spectra as described in the text.

Conclusion

Ion trap MS detection combined with PGC-LC provides an ideal solution for detailed characterization of mucin type *O*-glycans in highly complex mixtures. MS^2 spectra allow an easy differentiation and recognition of isobaric *O*-glycans. When using diagnostic cross-ring cleavages obtained in targeted MS^3 analyses - which is one of the unique features of ion trap MS - even higher levels of confidence in structural details are achieved.

Abbreviations

PGC = Porous Graphitized Carbon
RT = Retention Time
LC = Liquid Chromatography
SPS = Smart Parameter Setting
ICC = Ion Charge Control

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