Selective linkage detection of *O*-sialoglycan isomers by negative electrospray ionization-ion trap tandem mass spectrometry

by

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Abstract

Sialylated *O*-linked oligosaccharides are involved in many biological processes, such as cell-cell interactions, cell-substance adhesion, and virus-host interactions. These activities depend on their structure, which is frequently determined by tandem mass spectrometry. However, these spectra are frequently analyzer dependent, which makes difficult to develop widely applicable analytical methods. In order to deepen the origin of this behavior, two couples of isomers of sialylated *O*-linked oligosaccharides, NeuAcα2-3Galβ1-3GalNAc-ol / Galβ1-3(NeuAcα2-6)GalNAc-ol and NeuGcα2-3Galβ1-3GalNAc-ol / Galβ1-3(NeuGcα2-6)GalNAc-ol, were analyzed by LC-ESI(−)-MS^n using both an ion trap and a triple quadrupole mass spectrometers. Results clearly showed that while ions obtained in the triple quadrupole instrument fitted very well with the standard fragmentation routes, in the ion trap several intense ions could not be explained by these rules, specially a fragment at *m/z* 597. Furthermore, this ion was observed in the mass spectrum of those isomers that sialic acid binds to GalNAc by an α2,6-linkage. From the MS^3 spectrum of this ion an unexpected structure was deduced, and it led to propose alternative fragmentation pathways. Molecular mechanics calculations suggested that the found atypical route could be promoted by a hydrogen bond located only in α2-6-linked oligosaccharides. It has also been demonstrated that this process follows a slow kinetic, explaining why it cannot be observed using an ion beam-type mass analyzer. In conclusion, ion traps seem to be more appropriated than triple quadrupoles to develop a reliable analytical method to distinguish between isomeric *O*-linked glycans.

INTRODUCTION

O-glycans are formed by glycosylation of the hydroxyl oxygen of serine or threonine side chains generally in sequence regions of high hydroxyamino acid density. The *O*-linked carbohydrates found most commonly on secreted and membrane glycoproteins are attached to the peptide core through *N*-acetylgalactosamine (GalNAc). Sialic acids are typically found to be terminating branches of *O*-glycans, *N*-glycans and gangliosides, being the most common linkages to the C-3 or C-6 positions of galactose (Gal) residues or to the C-6 position of GalNAc residues[1]. Sialic acids also contain a carboxylic acid group at the C-1 position that is typically ionized at physiological pH. Because of their terminal position and negative charge, sialic acids play essential regulatory and protective roles in cell biology, such as ion transport, stabilization of protein conformation, protection from proteolytic attack, regulation of immune responses, modulation of receptor affinity and transmembrane signalling, and component of receptors or masking of recognition sites, thus, regulating molecular and cellular interactions[2].

The distribution of sialic acids in nature and their pronounced chemical diversity contribute to the enormous structural diversity of glycan chains, and, consequently, to the biological role exerted by the sialoglycan chain. In this sense, the type of sialic acids linkage (i.e., α2-3 or α2-6) has shown to be critical in the transmission of a number of viruses from animals to humans or other mammals[3]. Thus, while avian influenza viruses preferentially use α2-3-linked sialic acids[4], human influenza A viruses specifically bind to sialic acid in α2-6-linkage[5], and swine influenza viruses, are reported to bind sialic acid in either α2-6- or both α2-3- and α2-6-linkages[6]. Furthermore, mammalian sialic-acid-recognizing receptors (Siglec family of lectins) may also vary in their recognition of different sialic acids and can distinguish between glycosydic linkages. As an example, sialoadhesin and myelin-associated glycoprotein (MAG) only bind to α2-3-linked sialic acids, while cluster of differentiation-22 (CD22) recognizes α2-6 bonds[2].

Therefore, in order to fully understand the potential role of *O*-sialoglycans, it is of paramount importance to develop reliable techniques which allow their exhaustive characterization and the monitorization of changes in their structures which may parallel changes in the biological function of glycoproteins. Traditionally, determination of the linkage position involves the use of specific enzymes (sialidases)[7] or pre-derivatizing treatments[8]. Nevertheless, some sialic acid linkages may be partially or completely resistant to certain sialidases, while some

substitutions are particularly labile (e.g., *O*-acetylation) and/or can alter the behaviour of sialic acids during release, purification, and pre-treatment analysis[1]. For these reasons, nowadays, these procedures are being replaced by methods which allow a more direct analysis of the sample. As it has been shown in previous reviews[9-12], in the last decade mass spectrometry (MS) has proved to be one of the most important techniques for the analysis of oligosaccharides, being particularly useful when working in the tandem mode (MS^n), as it allows to obtain information on sequence and linkage position. In this sense, there are numerous papers where sialylated oligosaccharides are analyzed employing different combinations of ionization techniques and mass analyzers. Among ionization techniques, matrix-assisted laser desorption/ionization (MALDI) and electrospray ionization (ESI) are the most prevalent ones. Regarding the mass analyzer, triple quadrupole and quadrupole/time-of-flight are the ion beam-type designs more frequently used, and the Paul and Penning's traps among the ion trap-type[13-16].

It is well known that the fragmentation pattern in MS and tandem MS depend on several factors, namely on ionization techniques (electron impact ionization, ESI, etc.), on the use of positive or negative ions[17,18], on the different fragmentation conditions, and also on the mass analyzer design. This may be applied to any compound, including oligosaccharides.

The present work studies the fragmentation pattern of two couples of isomeric sialylated *O*-linked oligosaccharides containing *N*-acetylneuraminic (NeuAc) or *N*-glycolylneuraminic (NeuGc) acids, analyzed by high-performance liquid chromatography followed by negative electrospray ionization and tandem mass spectrometry (LC-ESI(–)-MS^n) using both an ion trap and a triple quadrupole mass spectrometers. Special attention is paid to the generation of mass fragments from *O*-sialoglycans that are not explained by the conventional rules[19], when low energy tandem mass spectra are obtained with an ion trap mass analyzer. In addition, the fragmentation pattern of a sialylated tetrasaccharide containing both α2-3 and α2-6 linked NeuGc residues was also elucidated.

EXPERIMENTAL

Chemicals

Bovine milk was provided by a dairy farm (Campo Real, Madrid, Spain). Freshly drawn equine blood was provided by the municipal abattoir of Mieres (Asturias, Spain). The

chemicals used for sample treatment were of analytical grade (Merck, Darmstadt, Germany). Commercial rennet powder, containing 85% chymosin (EC 3.4.23.4) and 15% bovine pepsin (EC 3.4.23.1), was obtained from Chr. Hansen′s Laboratorium (DK-1250 Copenhagen, Denmark). Resin Dowex 50W-X2 was from Sigma-Aldrich (St. Louis, MO, USA). For liquid chromatography experiments, acetonitrile of LC-MS Chromasolv grade (Riedel-de Haën, Seelz, Germany), ammonium acetate of analytical grade (Merck) and Milli-Q water obtained using a Millipore (Bedfor, USA) system were used.

Standards preparation

Oligosaccharides used in this study, whose chemical structures are known from previous works[20,21], were isolated from horse glycophorins and bovine caseinomacropeptide (CMP) as follows.

Erythrocyte ghosts were prepared from freshly drawn equine blood, mixed with 0.2 M EDTA (25:1, v/v), then diluted with an equal volume of cold 320 mOsm sodium phosphate buffer (pH 7.4) (160 mM NaH_2PO_4 adjusted to pH 7.4 with 107 mM Na_2HPO_4). Erythrocytes were sedimented at 500 *g* for 10 min at 4 ºC, and plasma and buffy coat removed. The cells were washed three times with the same buffer and subsequently were lysed by mixing with 30-40 volumes of 20 mOsm sodium phosphate buffer (pH 7.4), as described by Fairbanks *et al.*[22]. After lyophilization, erythrocyte ghosts were delipidated by extraction twice with chloroform-methanol (2:1, v/v) and four times with chloroform-methanol (1:2, v/v)[23]. The delipidated ghosts were dried and stored at -18 ºC, before further analyses.

To obtain CMP, whole casein was prepared by precipitation from bovine skim milk by adjusting the pH to 4.6 with 1 M HCl, followed by centrifugation at 4500 *g* and 5 ºC for 15 min. The casein precipitate was washed three times with 1 M sodium acetate acetic acid buffer, pH 4.6, thoroughly dialyzed against water and lyophilized. Rennet solution (1 mL, 4 mg mL^{-1}) was added to bovine casein solution (25 g L^{-1}) in 0.1 M sodium phosphate buffer, pH 6.5 (100 mL) and the mixture incubated at 35 ºC for 20 min. To inactivate chymosin, 0.2 M NaOH was added to pH 9.0–9.5, followed by heating at 60 ºC for 15 min[24]. The sample was adjusted to pH 4.6 with 1 M HCl and centrifuged at 4500 *g* and 5 ºC for 15 min. The supernatant was filtered through glass wool, subjected to exhaustive dialysis against water at 4 ºC and, finally, lyophilized.

O-linked oligosaccharides were liberated by *β*-elimination as follows: CMP (75 mg) or the delipidated ghosts (180 mg) were dissolved in 5 or 18 mL, respectively of 0.05 M NaOH containing 1 M $NaBH_4$ and incubated at 55 ºC overnight. The solution was acidified with acetic acid to remove the excess of borohydride. Methanol was added and methylborate was removed by evaporation in a vacuum. The residue was dissolved in water, and applied to a column of Dowex 50W X2 (H^+ form, 200 mesh) at 4 ºC. The mixture of oligosaccharide alditols eluted with water was evaporated in a vacuum to dryness.

Instrumental analysis

Two LC-MS instruments, both from Thermo Fisher Scientific (San Jose, CA, USA) were used in this study. The first one was an Accela pump with quaternary gradient system coupled to a TSQ Quantum Access triple quadrupole mass spectrometer using an ESI interface working in the negative mode. Sample injections were carried out by an Accela autosampler. Nitrogen (99.5% purity) was used as sheath and auxiliary gas, and argon (99.9990% purity) as the collision gas at 1.5 mTorr. Spray voltage was set at 3.5 kV, heated capillary temperature at 225 °C, sheath gas at 0.6 L min^{-1} and auxiliary gas at 6 L min^{-1}.

The second instrument was a Surveyor pump with quaternary gradient system coupled to a LCQ Deca ion trap mass spectrometer using an ESI interface. Sample injections were carried out by a Surveyor autosampler. Nitrogen (99.5% purity) was used as sheath and auxiliary gas, and helium (99.9990% purity) as the collision gas. Spray voltage was set at 4.5 kV, heated capillary temperature at 225 °C, sheath gas at 0.6 L min^{-1} and finally, auxiliary gas at 6 L min^{-1}. Mass spectra were acquired in the negative ion mode.

Optimization of both, ion transmission into the analyzer and tandem MS parameters was performed by infusing a mixture of oligosaccharides derived from bovine CMP (10 µg $µL^{-1}$) at a flow rate of 5 µL min^{-1} using the syringe pump included in the instrument and mixing it with 100 µL min^{-1} of acetonitrile:ammonium acetate 20 mM (50:50, v/v) by means a zero-dead volume T-piece. Collision energies for tandem MS experiments were chosen such as the intensity of the precursor ion remains at least as the 5% of the base peak in the MS^n spectrum. Separation of the oligosaccharide samples was performed on a Hypercarb column (100 x 2.1 mm), supplied by Thermo Fisher Scientific. Solvent A was 10 mM ammonium acetate, containing 2% acetonitrile; and solvent B 10 mM ammonium acetate, containing 80% acetonitrile. The gradient was developed from 0-50% of B over 40 min. The column was then

washed with 100% B for 5 min and re-equilibrated with the starting ratio of solvents for 20 min. A flow rate of 100 µL min^{-1} was used for every analysis, and a sample volume of 20 µL (100 ng/µL) was injected. All sample solutions were passed through a 0.45 µm nylon filter (Whatman Inc., Clifton, NJ, USA) before injection. Under the described analytical conditions, the tetrasaccharide and the isomers α2-3 and α2-6 from trisaccharides were baseline resolved (data not shown). Differentiation between the trisaccharides with the same type of linkage was achieved on the basis of their different *m/z* ratios.

Fragment annotations applied in this study were based on the nomenclature suggested by Domon and Costello[19], and extended by Karlsson *et* al.[25] for alditols.

The LC-MS system data acquisition and processing were managed by Xcalibur software (1.2 and 2.0 versions, Thermo Fisher Scientific).

Molecular mechanics calculations

Conformational energy calculations for ions were performed by applying the MM2 method implemented into the Chem3D Ultra program (version 11.0, CambridgeSoft Corporation, MA, USA). During the energy calculation, the negative charge was assumed to be localized on the carboxyl group of sialic acid, and all glycosidic bond angles were rotated.

RESULTS AND DISCUSSION

Study of isomeric trisaccharides with NeuAcα2-3 linked to Gal or NeuAcα2-6 linked to GalNAc-ol

In order to characterize the structure of these isomers isolated from animal samples, i. e. horse glycophorins and bovine CMP, LC-ESI(–)-MS^2 analyses with a triple quadrupole mass spectrometer were carried out. Figures 1(a) and 1(b) show the MS^2 spectra obtained from their respective precursor ions at *m/z* 675 ($[M–H]^-$). These MS^2 spectra were similar to those found in the literature, although we were unable to find some important diagnostic ions referred by other authors[26-29]. In this sense, Robbe *et al.*[26] studied the fragmentation of different isomeric sialylated *O*-linked oligosaccharides using a quadrupole/time-of-flight mass analyzer with an electrospray interface working in the negative mode. Among other oligosaccharides, NeuAcα2-3Galβ1-3GalNAc-ol and Galβ1-3(NeuAcα2-6)GalNAc-ol,

whose only structural difference is the sialyl α2-3 and α2-6 linkage (structures in Fig. 1), were analyzed. These authors developed a detailed structural elucidation of these trisaccharides from their MS^2 spectra, finding a diagnostic ion at *m/z* 408 ($B_2 - CO_2$) for the trisaccharide with the α2-3 linkage. For the trisaccharide with the α2-6 linkage, two characteristic ion were found at *m/z* 513 ($Y_{1\beta}$) and *m/z* 306 ($^4A_{0\alpha} - CO_2$), this last one also used by other authors[27,28]. Additionally, another ion found at *m/z* 454 ($^{0,2}X_{1\alpha}$ or $^{0,2}X_2$) in both compounds was significantly more intense for the trisaccharide with NeuAcα2-6 linked to GalNAc-ol. In any case, all the mentioned diagnostic ions showed a very low intensity (< 5%), which could be a problem if a differentiation of isomers at low concentrations is required. As it was in our case, Olson *et al.*[29] did not find any of the cited diagnostic ions, even though they analyzed the same compounds using the same type of mass analyzer. Perhaps the low intensity of the diagnostic ions and small differences in the experimental working conditions produced these divergences.

In spite of an exhaustive work on ionization and fragmentation conditions in our triple quadrupole mass spectrometer, the detection of ions at *m/z* 306, 408 or 513 was not accomplished, probably due to their low intensity. Nevertheless, the ion at *m/z* 454 was indeed detected at a low abundance, although this was not very useful since it was present in both isomers (Figs. 1(a) and 1(b)).

Bearing in mind the results obtained so far, we tried to acquire better spectra transferring the analytical method to another mass spectrometer equipped with an ion trap analyzer. Whilst elution conditions remained unchanged, those parameters influencing ionization, desolvatation and ion transmission from source to analyzer were re-optimized for the new analyzer. Strikingly, the mass spectra obtained for both isomers using the ion trap were noticeably different. Thus, the MS^2 spectrum of the trisaccharide with the α2-3 linkage was very similar to that obtained with the triple quadrupole (Fig. 1(c)), and its fragmentation process was in good agreement with that proposed by Domon and Costello[19]. Conversely, the MS^2 spectrum corresponding to the trisaccharide with the α2-6 linkage showed marked differences (Fig. 1(d)). Similar to Robbe *et al.*[26], a small diagnostic ion was observed at *m/z* 513, but the attention was attracted by two more intense ions at *m/z* 615 and 597 (labeled with an asterisk in Fig. 1(d)). In order to ascertain the quality of the spectrum and discard the presence of any interfering compound, blank analysis and high-resolution mass spectra of the precursor ion (*m/z* 675) were performed, without finding isobaric interferences (spectra not shown). The occurrence of artifacts generation during the MS^2 spectrum recording was ruled

out, as it was registered again after varying several parameters such as time and isolation width of precursor ion, activation q_z value and the amount of precursor ions trapped in the mass analyzer. As a result, no significant differences with the initial MS^2 spectrum were observed, indicating the good quality and reliability of the MS^2 spectra analyses.

Therefore, the next step was to deal with the structural elucidation of the main ions present in the MS^2 spectrum of the Galβ1-3(NeuAcα2-6)GalNAc-ol oligosaccharide. In this sense, the presence of fragments at *m/z* 615 and 597 might be explained by the pathway proposed in Scheme 1a. According to this fragmentation pathway, the ion at *m/z* 615 ($^{2,4}X_{1\beta}$ or $^{1,3}X_{1\beta}$ fragments) appears through the neutral loss of 60 mass units as $C_2H_4O_2$, and the ion at *m/z* 597 by its posterior unspecified dehydration.

In order to confirm this fragmentation mechanism, MS^3 experiments were performed. The ion at *m/z* 675 was, thus, broken and its product ions at *m/z* 615 and 597 were again used as precursors. It was observed that the ion at *m/z* 597 stemmed from ion 615 through the loss of water (MS^3 spectrum not shown), and that the MS^3 spectrum of ion *m/z* 597 presented a rich fragmentation (Fig. 2), in contrast to the scarcely populated MS^2 spectrum obtained from the original precursor (*m/z* 675). Among the resulting fragments, whose identity is discussed below, two facts should be pointed out. First of all, no traces of ions 290 ($B_{1\alpha}$) or 306 ($^4A_{0\alpha} - CO_2$) were detected (see enlarged detail in Fig. 2), allowing us to discard the pathway shown in Scheme 1a. Furthermore, an intense and revealing ion at *m/z* 384 ($Y_{1\alpha}$ or Y_2) was observed, indicating that the fragment lost in the transition 675 > 597 comes from the NeuAc unit. To the best of our knowledge, this process has not yet been described in the literature, and it leads to propose the alternative fragmentation route described in Scheme 1b. This new route is clearly supported by the capability of assigning all the main fragments of the MS^3 spectrum of the ion at *m/z* 597 ($^{4,5}X_{1\alpha} - H_2O$), as it is shown in Fig. 2.

To find an explanation, and taking into account the role that conformation plays in oligosaccharide fragmentation[30], minimum energy conformations were calculated for the ionized oligosaccharides using the MM2 method. Results are shown in Figs. 3(a) and 3(b), where only for the trisaccharide with the α2-6 linkage, the formation of a hydrogen bond between a hydroxyl group of the propanetriol branch sited on the NeuAc unit and the negatively charged oxygen could be observed. Thus, the spatial conformation may justify a proton transfer process between the two groups involved in this hydrogen bond, which would later on induct fragmentation in this area of the molecule, with a neutral loss of $C_2H_4O_2$. The

viability of this process is supported by a recent work of Zaia *et al.*[31], who studied the relevance of proton transfer processes in the negative ion fragmentation of oligosaccharides. On the other hand, it should be noted that the ion at m/z 597 is not observed using beam-type mass analyzers, suggesting that it comes from a slow kinetic process, and agreeing with the fragmentation pathways of Scheme 1b, that involves several rearrangements. To check this point, MS^2 spectra from ion at *m/z* 675 were obtained using activation times ranging from 1 to 1000 ms, and recording the intensities of ions found at *m/z* 597, 454 and 290. These last two ions were selected because are also detected with beam-type mass analyzers, and therefore, generated by a fast kinetic process. Figure 4(a) shows the intensities of ions at *m/z* 597 and 454, relativized to ion at *m/z* 290, against the activation time. As can be seen, the relative intensity of ion at *m/z* 454 remained almost unchanged (fast kinetic), while the ion at *m/z* 597 was very small for collision times below 3 ms, gradually increasing up to 25 ms and showing not significant changes above this value. Thus, this experiment confirm that the ion at *m/z* 597 is generated by a slow kinetic process, compatible with the pathway shown in Scheme 1b, and also justify its absence in mass spectra obtained with beam-type mass analyzers. In fact, a similar behavior has been described for peptides analyzed using an ion trap mass spectrometer[32,33].

Study of isomeric trisaccharides with NeuGcα2-3 linked to Gal or NeuGcα2-6 linked to GalNAc-ol

With the aim of confirming if this type of fragmentation is characteristic of the preceding oligosaccharides, the same methodology was applied to the following isomers containing NeuGc instead of NeuAc: NeuGcα2-3Galβ1-3GalNAc-ol and Galβ1-3(NeuGcα2-6)GalNAc-ol, and whose structures are displayed in Fig. 5. After the MS optimization process, MS^2 spectra of both oligosaccharides using the triple quadrupole (Figs. 5(a) and 5(b)) and the ion trap (Figs. 5(c) and 5(d)) analyzers were obtained from their respective precursor ions at *m/z* 691 ($[M–H]^-$). As far as we know, this is the first time that these trisaccharides have been detected and characterized by tandem mass spectrometry in their alditol form. Most of the recorded ions were easily assigned through usual fragmentation processes. However, the presence of a very intense ion at *m/z* 597 (60% of the base peak) belonging to the trisaccharide with the α2-6 linkage was observed again, but only in the spectrum obtained with the ion trap (Fig. 5(d)). More surprisingly, the fragmentation of this ion produced a MS^3

spectrum identical to the one shown in Fig. 2. This indicates that this ion (*m/z* 597) is directly derived from a loss in the NeuGc unit and that its structure should be identical to that of the ion at *m/z* 597 ($^{4,5}X_{1\alpha}$ – H_2O) coming from the trisaccharide containing NeuAc. Three-dimensional representations optimized through the MM2 method, and presented in Figs. 3(c) and 3(d), also revealed a hydrogen bond between a hydroxyl group of the propanetriol branch sited on the NeuGc unit and the negatively charged oxygen for the trisaccharide with the α2-6 linkage. As it was the case for NeuAc, such interaction was not found in the trisaccharide with the α2-3 linkage. A tentative fragmentation pathway is shown in Scheme 2, where it is proposed the formation of a cycle as a reaction intermediate, which later on breaks and pursues the described process. The stability and viability of this cycle were also supported by the performed spatial structure calculations.

Likewise, the described process should imply a slow kinetic, which was confirmed with the same type of experiment that was carried out for the oligosaccharide with NeuAc, but using the ion at *m/z* 691 as precursor and recording ions at *m/z* 597, 454 and 306 ($B_{1\alpha}$). It can be seen in Fig. 4(b) that the intensity of ion at *m/z* 454 (relative to ion at *m/z* 306) was almost constant with the activation time, so it should be generated by a fast kinetic process. On the contrary, the ion at *m/z* 597 was very weak below 3 ms and intense only above 25 ms, indicating a slow kinetic process. As it occurred with the NeuAc oligosaccharide, the slow kinetic generation route for ion at *m/z* 597 is compatible with the pathway shown in Scheme 2, and therefore, it would explain the difficulty in its detection by beam-type mass analyzers. The stability and viability of this route have also been supported by the spatial structure calculations carried out, and it is equivalent to the processes found by other authors[31,33].

Study of NeuGcα2-3Galβ1-3(NeuGcα2-6)GalNAc-ol

Once shown the fragmentation behavior of sialylated trisaccharides, the same type of study was carried out on a tetrasaccharide. The aim of this experiment was to investigate the possible use of fragment at *m/z* 597 as a diagnostic ion, not only for trisaccharides containing sialic acids bound to GalNAc by an α2,6-linkage, but also for longer oligosaccharides. Using the same experimental conditions, the tetrasaccharide NeuGcα2-3Galβ1-3(NeuGcα2-6)GalNAc-ol isolated from equine glycophorines was analyzed by LC-ESI(–)-MS^n with an ion trap mass spectrometer. Its $[M–H]^-$ ion (*m/z* 998) was used as precursor to obtain the MS^2 spectrum shown in Fig. 6(a), where the only significant ion detected corresponds to *m/z* 691,

coming from the lost of a NeuGc unit. As there is one NeuGc unit at both ends of the oligosaccharide chain, it is not possible at this point to know which one is lost. To answer this question, it was necessary to break the ion at *m/z* 691 ($Y_{2\alpha}$ or $Y_{1\beta}$) looking for the corresponding diagnostic ions. The resulting MS^3 spectrum is shown in Fig. 6(b), being obvious an intense ion at *m/z* 597. This ion was subjected to a further fragmentation step, obtaining a MS^4 spectrum identical to the one shown in Fig. 2, which indicates the presence of the Galβ1-3(NeuGcα2-6)GalNAc-ol in the tetrasaccharide. However, the existence of the ion at *m/z* 647 ($[M–H]^- - CO_2$) in the MS^3 spectrum also reveals the presence of the NeuGcα2-3Galβ1-3GalNAc-ol in the tetrasaccharide (see Fig. 5(c)). Therefore, the results indicate that the ion at *m/z* 691 comes from the mixture of the $Y_{2\alpha}$ and $Y_{1\beta}$ fragments.

In short, the tandem mass spectra obtained for this tetrasaccharide reveal that the first breaking process (MS^2) affect the glycosidic bond of NeuGc molecules, generating an ion at *m/z* 691 ($Y_{2\alpha}$ and $Y_{1\beta}$) whose MS^3 spectrum contains characteristics ions from trisaccharides with α2-3 (*m/z* 647) and α2-6 (*m/z* 597) linkages. Consequently, the new diagnostic ion at *m/z* 597 seems to be useful not only to elucidate the structure of sialylated trisaccharides but also for higher sialylated oligosaccharides.

CONCLUSIONS

The results presented in this work clearly show differences among fragmentation processes for α2-6-linked oligosaccharides when ion trap or triple quadrupole analyzers are used. They can be explained bearing in mind how ion traps work, since product ions are not affected by collision voltages, so second and third generation ruptures are not promoted, as it happens with beam-type instruments. Furthermore, they use long collision times, making easier to see slow kinetic reactions, such as molecular rearrangements. These facts have permitted us to describe new fragmentation pathways for the *O*-sialoglycans studied, that do not follow the standard processes described by Domon and Costello[19], and that seem to take relevance only when ion trap instruments are used. For all above, ion traps seem to be more appropriated than triple quadrupoles to develop an analytical method capable of distinguishing between isomeric *O*-linked glycans since a very intense diagnostic ion at *m/z* 597 is detected for molecules containing sialic acids bound to GalNAc by an α2,6-linkage.

Finally, it is recognized that glycans affect the activities of the protein to which they are attached by modulating their functions. Consequently, the developed ion trap multistage mass spectrometric method might be particularly important in the glycobiology field in order to fully understand the potential role of *O*-sialoglycan isomers, as the linkage position of sialic acid groups may involve crucial changes in the biological function of glycoproteins. In this field, advances in mass spectrometry instrumentation are being of great utilility for gaining better understanding of the molecular basis of the function of glycoproteins in biological processes.

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FIGURE LEGENDS

Figure 1. Chemical structures and MS^2 spectra of NeuAcα2-3Galβ1-3GalNAc-ol and Galβ1-3(NeuAcα2-6)GalNAc-ol (from bovine CMP) obtained in a triple quadrupole (a and b, respectively) and an ion trap (c and d, respectively) mass analyzers. In all cases, precursor ion was the *m/z* 675.

Figure 2. MS^3 spectrum of the *m/z* 597 fragment ion resulting from MS^2 of *m/z* 675. An enlarged detail of the low *m/z* interval is at the left corner.

Figure 3. Conformations of (a) NeuAcα2-3Galβ1-3GalNAc-ol, (b) Galβ1-3(NeuAcα2-6)GalNAc-ol, (c) NeuGcα2-3Galβ1-3GalNAc-ol and (d) Galβ1-3(NeuGcα2-6)GalNAc-ol. The hydrogen bond between the carbonyl and a hydroxyl group is shown as a dashed line.

Figure 4. Ion ratios obtained for (a) Galβ1-3(NeuAcα2-6)GalNAc-ol, and (b) Galβ1-3(NeuGcα2-6)GalNAc-ol using precursor ions at *m/z* 675 and *m/z* 691, respectively.

Figure 5. Chemical structures and MS^2 spectra of NeuGcα2-3Galβ1-3GalNAc-ol and Galβ1-3(NeuGcα2-6)GalNAc-ol (from equine glycophorines) obtained in a triple quadrupole (a and b, respectively) and an ion trap (c and d, respectively) mass analyzers. In all cases, precursor ion is the *m/z* 691.

Figure 6. Chemical structure, MS^2 (a) and MS^3 (b) spectra of NeuGcα2-3Galβ1-3(NeuGcα2-6)GalNAc-ol (from equine glycophorines) obtained in an ion trap mass analyzer.

Scheme 1. Proposed MS^2 pathways for Galβ1-3(NeuAcα2-6)GalNAc-ol.

Scheme 2. Proposed MS^2 pathway for Galβ1-3(NeuGcα2-6)GalNAc-ol.

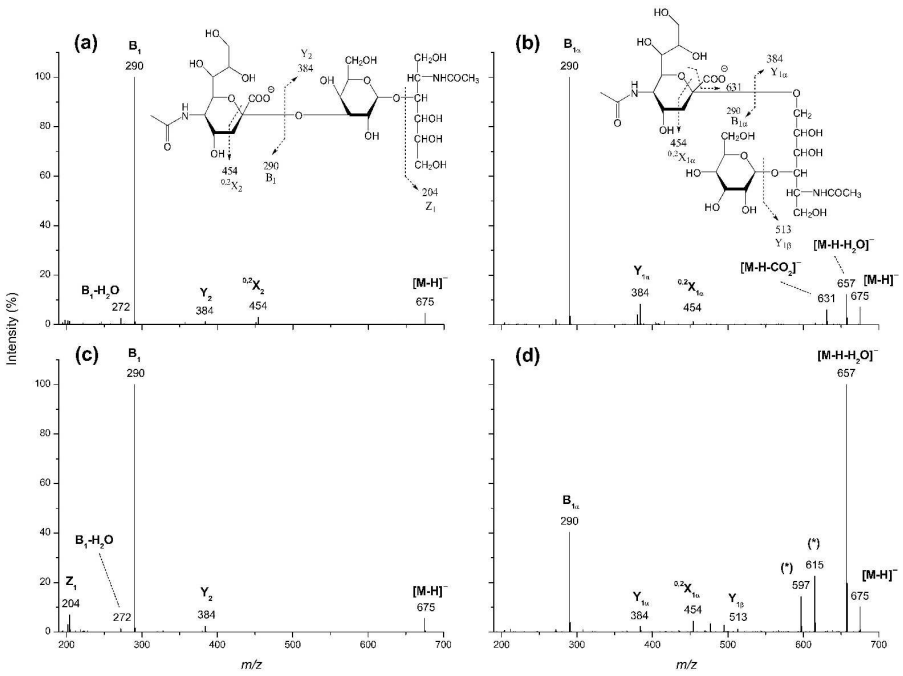

Figure 1
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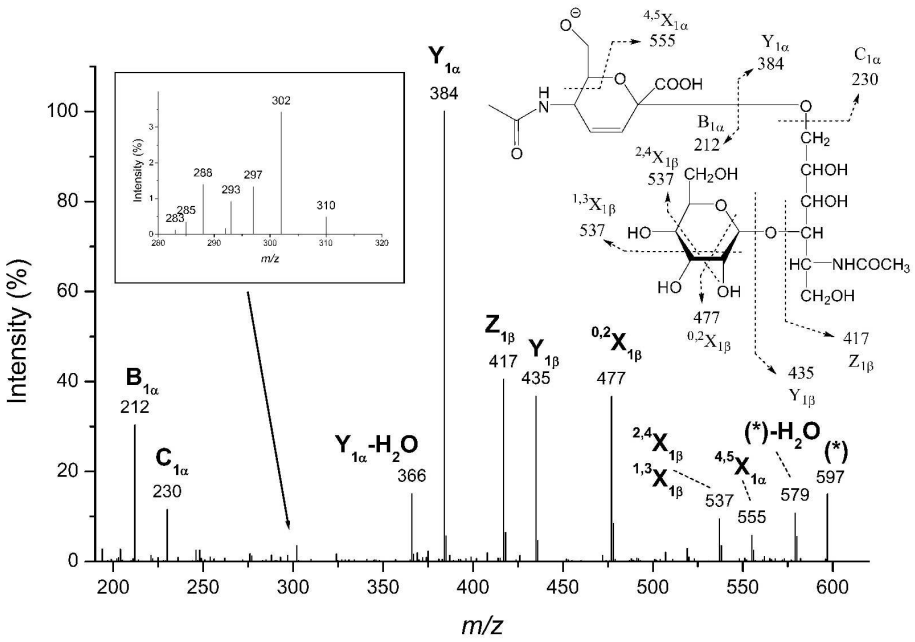

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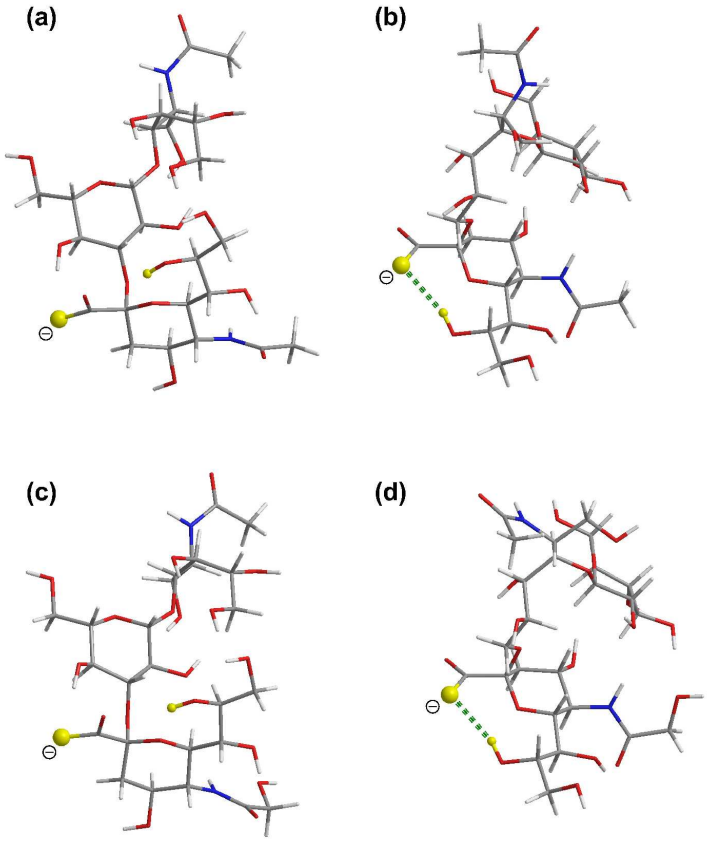

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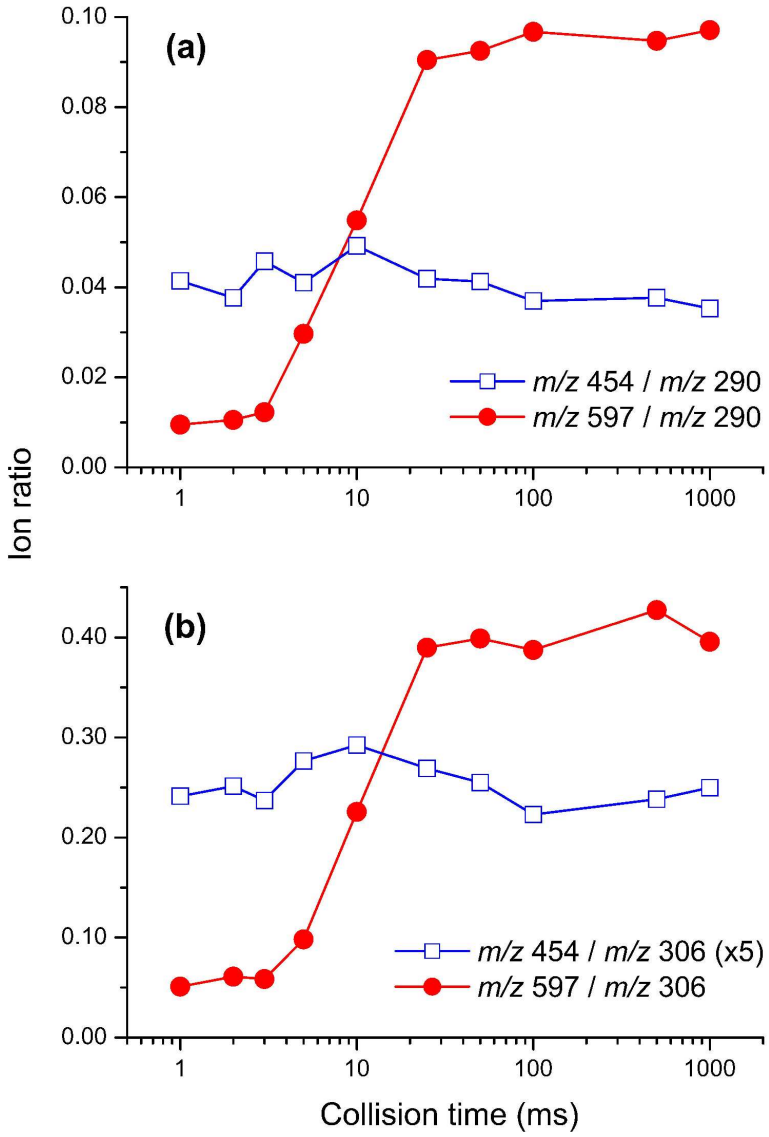

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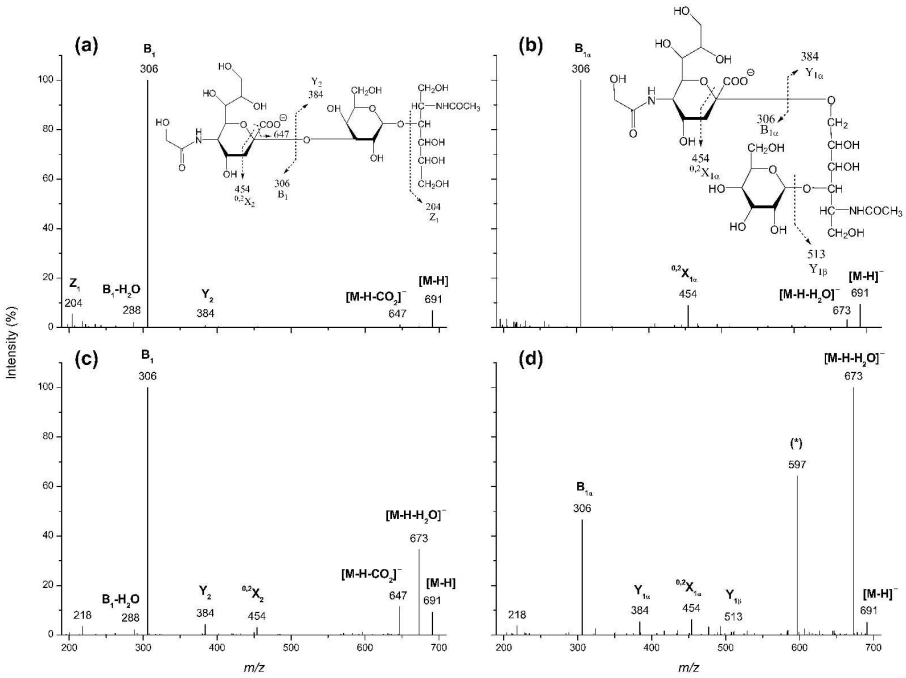

Figure 5
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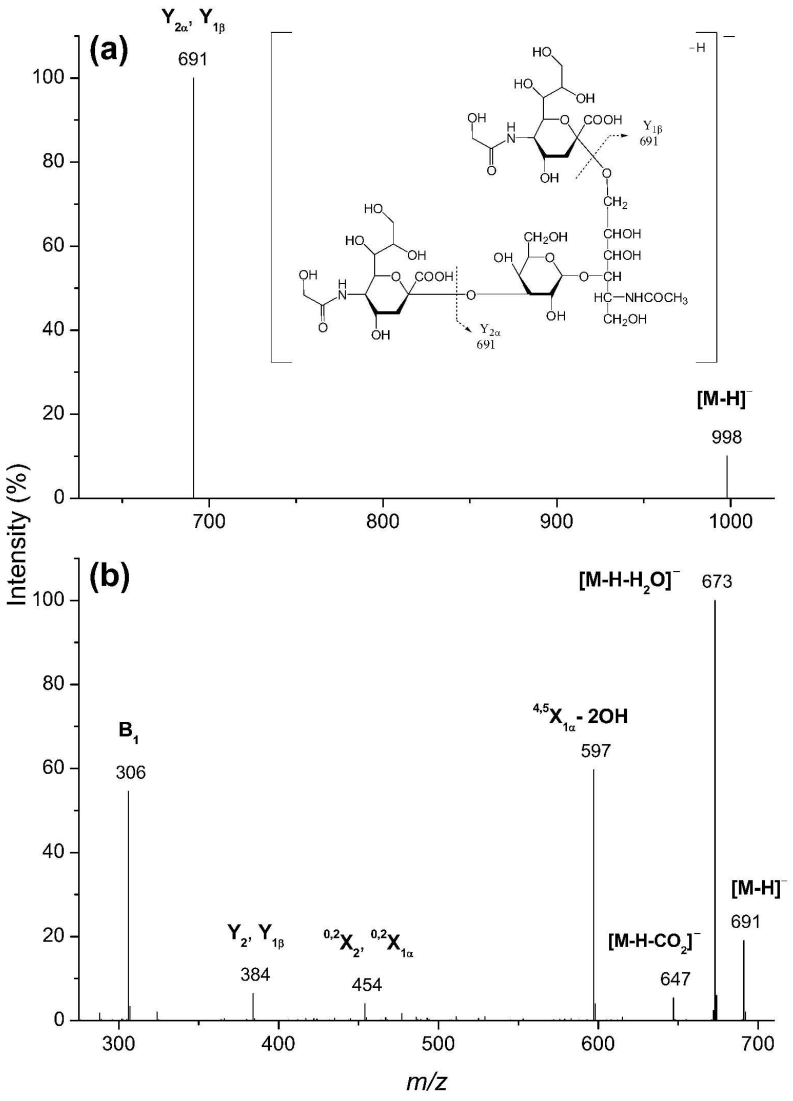

Figure 6
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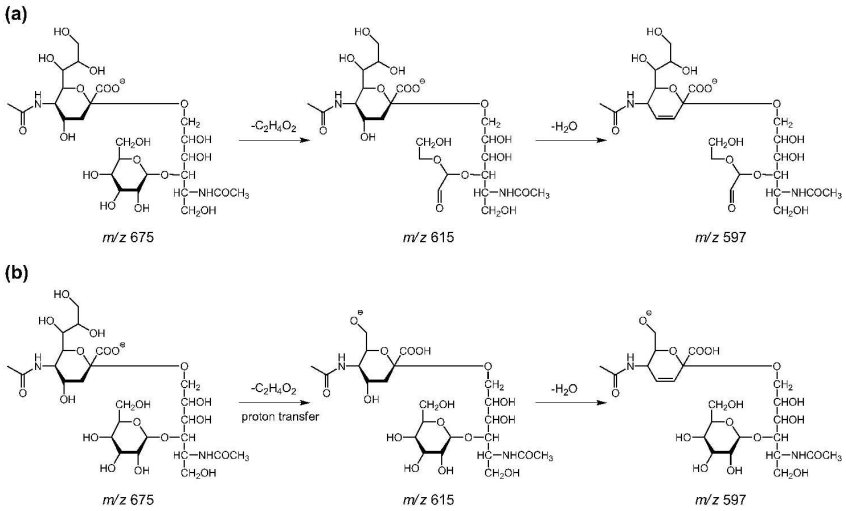

Scheme 1
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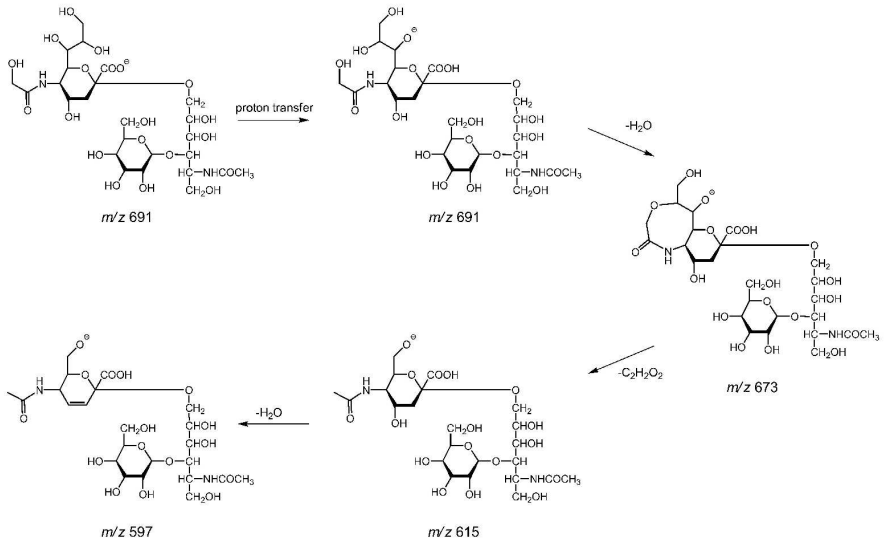

Scheme 2
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Selective linkage detection of *O*-sialoglycan isomers by negative electrospray ionization-ion trap tandem mass spectrometry

by

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Running Head

Fragmentation of *O*-linked oligosaccharides

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Abstract

Sialylated *O*-linked oligosaccharides are involved in many biological processes, such as cell-cell interactions, cell-substance adhesion, and virus-host interactions. These activities depend on their structure, which is frequently determined by tandem mass spectrometry. However, these spectra are frequently analyzer dependent, which makes difficult to develop widely applicable analytical methods. In order to deepen the origin of this behavior, two couples of isomers of sialylated *O*-linked oligosaccharides, NeuAcα2-3Galβ1-3GalNAc-ol / Galβ1-3(NeuAcα2-6)GalNAc-ol and NeuGcα2-3Galβ1-3GalNAc-ol / Galβ1-3(NeuGcα2-6)GalNAc-ol, were analyzed by LC-ESI(−)-MS^n using both an ion trap and a triple quadrupole mass spectrometers. Results clearly showed that while ions obtained in the triple quadrupole instrument fitted very well with the standard fragmentation routes, in the ion trap several intense ions could not be explained by these rules, specially a fragment at *m/z* 597. Furthermore, this ion was observed in the mass spectrum of those isomers that sialic acid binds to GalNAc by an α2,6-linkage. From the MS^3 spectrum of this ion an unexpected structure was deduced, and it led to propose alternative fragmentation pathways. Molecular mechanics calculations suggested that the found atypical route could be promoted by a hydrogen bond located only in α2-6-linked oligosaccharides. It has also been demonstrated that this process follows a slow kinetic, explaining why it cannot be observed using an ion beam-type mass analyzer. In conclusion, ion traps seem to be more appropriated than triple quadrupoles to develop a reliable analytical method to distinguish between isomeric *O*-linked glycans.

INTRODUCTION

O-glycans are formed by glycosylation of the hydroxyl oxygen of serine or threonine side chains generally in sequence regions of high hydroxyamino acid density. The *O*-linked carbohydrates found most commonly on secreted and membrane glycoproteins are attached to the peptide core through *N*-acetylgalactosamine (GalNAc). Sialic acids are typically found to be terminating branches of *O*-glycans, *N*-glycans and gangliosides, being the most common linkages to the C-3 or C-6 positions of galactose (Gal) residues or to the C-6 position of GalNAc residues[1]. Sialic acids also contain a carboxylic acid group at the C-1 position that is typically ionized at physiological pH. Because of their terminal position and negative charge, sialic acids play essential regulatory and protective roles in cell biology, such as ion transport, stabilization of protein conformation, protection from proteolytic attack, regulation of immune responses, modulation of receptor affinity and transmembrane signalling, and component of receptors or masking of recognition sites, thus, regulating molecular and cellular interactions[2].

The distribution of sialic acids in nature and their pronounced chemical diversity contribute to the enormous structural diversity of glycan chains, and, consequently, to the biological role exerted by the sialoglycan chain. In this sense, the type of sialic acids linkage (i.e., α2-3 or α2-6) has shown to be critical in the transmission of a number of viruses from animals to humans or other mammals[3]. Thus, while avian influenza viruses preferentially use α2-3-linked sialic acids[4], human influenza A viruses specifically bind to sialic acid in α2-6-linkage[5], and swine influenza viruses, are reported to bind sialic acid in either α2-6- or both α2-3- and α2-6-linkages[6]. Furthermore, mammalian sialic-acid-recognizing receptors (Siglec family of lectins) may also vary in their recognition of different sialic acids and can distinguish between glycosydic linkages. As an example, sialoadhesin and myelin-associated glycoprotein (MAG) only bind to α2-3-linked sialic acids, while cluster of differentiation-22 (CD22) recognizes α2-6 bonds[2].

Therefore, in order to fully understand the potential role of *O*-sialoglycans, it is of paramount importance to develop reliable techniques which allow their exhaustive characterization and the monitorization of changes in their structures which may parallel changes in the biological function of glycoproteins. Traditionally, determination of the linkage position involves the use of specific enzymes (sialidases)[7] or pre-derivatizing treatments[8]. Nevertheless, some sialic acid linkages may be partially or completely resistant to certain sialidases, while some

substitutions are particularly labile (e.g., *O*-acetylation) and/or can alter the behaviour of sialic acids during release, purification, and pre-treatment analysis[1]. For these reasons, nowadays, these procedures are being replaced by methods which allow a more direct analysis of the sample. As it has been shown in previous reviews[9-12], in the last decade mass spectrometry (MS) has proved to be one of the most important techniques for the analysis of oligosaccharides, being particularly useful when working in the tandem mode (MS^n), as it allows to obtain information on sequence and linkage position. In this sense, there are numerous papers where sialylated oligosaccharides are analyzed employing different combinations of ionization techniques and mass analyzers. Among ionization techniques, matrix-assisted laser desorption/ionization (MALDI) and electrospray ionization (ESI) are the most prevalent ones. Regarding the mass analyzer, triple quadrupole and quadrupole/time-of-flight are the ion beam-type designs more frequently used, and the Paul and Penning's traps among the ion trap-type[13-16].

It is well known that the fragmentation pattern in MS and tandem MS depend on several factors, namely on ionization techniques (electron impact ionization, ESI, etc.), on the use of positive or negative ions[17,18], on the different fragmentation conditions, and also on the mass analyzer design. This may be applied to any compound, including oligosaccharides.

The present work studies the fragmentation pattern of two couples of isomeric sialylated *O*-linked oligosaccharides containing *N*-acetylneuraminic (NeuAc) or *N*-glycolylneuraminic (NeuGc) acids, analyzed by high-performance liquid chromatography followed by negative electrospray ionization and tandem mass spectrometry (LC-ESI(–)-MS^n) using both an ion trap and a triple quadrupole mass spectrometers. Special attention is paid to the generation of mass fragments from *O*-sialoglycans that are not explained by the conventional rules[19], when low energy tandem mass spectra are obtained with an ion trap mass analyzer. In addition, the fragmentation pattern of a sialylated tetrasaccharide containing both α2-3 and α2-6 linked NeuGc residues was also elucidated.

EXPERIMENTAL

Chemicals

Bovine milk was provided by a dairy farm (Campo Real, Madrid, Spain). Freshly drawn equine blood was provided by the municipal abattoir of Mieres (Asturias, Spain). The

chemicals used for sample treatment were of analytical grade (Merck, Darmstadt, Germany). Commercial rennet powder, containing 85% chymosin (EC 3.4.23.4) and 15% bovine pepsin (EC 3.4.23.1), was obtained from Chr. Hansen′s Laboratorium (DK-1250 Copenhagen, Denmark). Resin Dowex 50W-X2 was from Sigma-Aldrich (St. Louis, MO, USA). For liquid chromatography experiments, acetonitrile of LC-MS Chromasolv grade (Riedel-de Haën, Seelz, Germany), ammonium acetate of analytical grade (Merck) and Milli-Q water obtained using a Millipore (Bedfor, USA) system were used.

Standards preparation

Oligosaccharides used in this study, whose chemical structures are known from previous works[20,21], were isolated from horse glycophorins and bovine caseinomacropeptide (CMP) as follows.

Erythrocyte ghosts were prepared from freshly drawn equine blood, mixed with 0.2 M EDTA (25:1, v/v), then diluted with an equal volume of cold 320 mOsm sodium phosphate buffer (pH 7.4) (160 mM NaH_2PO_4 adjusted to pH 7.4 with 107 mM Na_2HPO_4). Erythrocytes were sedimented at 500 *g* for 10 min at 4 ºC, and plasma and buffy coat removed. The cells were washed three times with the same buffer and subsequently were lysed by mixing with 30-40 volumes of 20 mOsm sodium phosphate buffer (pH 7.4), as described by Fairbanks *et al*.[22]. After lyophilization, erythrocyte ghosts were delipidated by extraction twice with chloroform-methanol (2:1, v/v) and four times with chloroform-methanol (1:2, v/v)[23]. The delipidated ghosts were dried and stored at -18 ºC, before further analyses.

To obtain CMP, whole casein was prepared by precipitation from bovine skim milk by adjusting the pH to 4.6 with 1 M HCl, followed by centrifugation at 4500 *g* and 5 ºC for 15 min. The casein precipitate was washed three times with 1 M sodium acetate acetic acid buffer, pH 4.6, thoroughly dialyzed against water and lyophilized. Rennet solution (1 mL, 4 mg mL^{-1}) was added to bovine casein solution (25 g L^{-1}) in 0.1 M sodium phosphate buffer, pH 6.5 (100 mL) and the mixture incubated at 35 ºC for 20 min. To inactivate chymosin, 0.2 M NaOH was added to pH 9.0–9.5, followed by heating at 60 ºC for 15 min[24]. The sample was adjusted to pH 4.6 with 1 M HCl and centrifuged at 4500 *g* and 5 ºC for 15 min. The supernatant was filtered through glass wool, subjected to exhaustive dialysis against water at 4 ºC and, finally, lyophilized.

O-linked oligosaccharides were liberated by *β*-elimination as follows: CMP (75 mg) or the delipidated ghosts (180 mg) were dissolved in 5 or 18 mL, respectively of 0.05 M NaOH containing 1 M $NaBH_4$ and incubated at 55 ºC overnight. The solution was acidified with acetic acid to remove the excess of borohydride. Methanol was added and methylborate was removed by evaporation in a vacuum. The residue was dissolved in water, and applied to a column of Dowex 50W X2 (H^+ form, 200 mesh) at 4 ºC. The mixture of oligosaccharide alditols eluted with water was evaporated in a vacuum to dryness.

Instrumental analysis

Two LC-MS instruments, both from Thermo Fisher Scientific (San Jose, CA, USA) were used in this study. The first one was an Accela pump with quaternary gradient system coupled to a TSQ Quantum Access triple quadrupole mass spectrometer using an ESI interface working in the negative mode. Sample injections were carried out by an Accela autosampler. Nitrogen (99.5% purity) was used as sheath and auxiliary gas, and argon (99.9990% purity) as the collision gas at 1.5 mTorr. Spray voltage was set at 3.5 kV, heated capillary temperature at 225 °C, sheath gas at 0.6 L min^{-1} and auxiliary gas at 6 L min^{-1}.

The second instrument was a Surveyor pump with quaternary gradient system coupled to a LCQ Deca ion trap mass spectrometer using an ESI interface. Sample injections were carried out by a Surveyor autosampler. Nitrogen (99.5% purity) was used as sheath and auxiliary gas, and helium (99.9990% purity) as the collision gas. Spray voltage was set at 4.5 kV, heated capillary temperature at 225 °C, sheath gas at 0.6 L min^{-1} and finally, auxiliary gas at 6 L min^{-1}. Mass spectra were acquired in the negative ion mode.

Optimization of both, ion transmission into the analyzer and tandem MS parameters was performed by infusing a mixture of oligosaccharides derived from bovine CMP (10 µg $µL^{-1}$) at a flow rate of 5 µL min^{-1} using the syringe pump included in the instrument and mixing it with 100 µL min^{-1} of acetonitrile:ammonium acetate 20 mM (50:50, v/v) by means a zero-dead volume T-piece. Collision energies for tandem MS experiments were chosen such as the intensity of the precursor ion remains at least as the 5% of the base peak in the MS^n spectrum. Separation of the oligosaccharide samples was performed on a Hypercarb column (100 x 2.1 mm), supplied by Thermo Fisher Scientific. Solvent A was 10 mM ammonium acetate, containing 2% acetonitrile; and solvent B 10 mM ammonium acetate, containing 80% acetonitrile. The gradient was developed from 0-50% of B over 40 min. The column was then

washed with 100% B for 5 min and re-equilibrated with the starting ratio of solvents for 20 min. A flow rate of 100 µL min^{-1} was used for every analysis, and a sample volume of 20 µL (100 ng/µL) was injected. All sample solutions were passed through a 0.45 µm nylon filter (Whatman Inc., Clifton, NJ, USA) before injection. Under the described analytical conditions, the tetrasaccharide and the isomers α2-3 and α2-6 from trisaccharides were baseline resolved (data not shown). Differentiation between the trisaccharides with the same type of linkage was achieved on the basis of their different *m/z* ratios.

Fragment annotations applied in this study were based on the nomenclature suggested by Domon and Costello[19], and extended by Karlsson *et* al.[25] for alditols.

The LC-MS system data acquisition and processing were managed by Xcalibur software (1.2 and 2.0 versions, Thermo Fisher Scientific).

Molecular mechanics calculations

Conformational energy calculations for ions were performed by applying the MM2 method implemented into the Chem3D Ultra program (version 11.0, CambridgeSoft Corporation, MA, USA). During the energy calculation, the negative charge was assumed to be localized on the carboxyl group of sialic acid, and all glycosidic bond angles were rotated.

RESULTS AND DISCUSSION

Study of isomeric trisaccharides with NeuAcα2-3 linked to Gal or NeuAcα2-6 linked to GalNAc-ol

In order to characterize the structure of these isomers isolated from animal samples, i. e. horse glycophorins and bovine CMP, LC-ESI(−)-MS^2 analyses with a triple quadrupole mass spectrometer were carried out. Figures 1(a) and 1(b) show the MS^2 spectra obtained from their respective precursor ions at *m/z* 675 ($[M-H]^-$). These MS^2 spectra were similar to those found in the literature, although we were unable to find some important diagnostic ions referred by other authors[26-29]. In this sense, Robbe *et al.*[26] studied the fragmentation of different isomeric sialylated *O*-linked oligosaccharides using a quadrupole/time-of-flight mass analyzer with an electrospray interface working in the negative mode. Among other oligosaccharides, NeuAcα2-3Galβ1-3GalNAc-ol and Galβ1-3(NeuAcα2-6)GalNAc-ol,

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whose only structural difference is the sialyl α2-3 and α2-6 linkage (structures in Fig. 1), were analyzed. These authors developed a detailed structural elucidation of these trisaccharides from their MS^2 spectra, finding a diagnostic ion at *m/z* 408 ($B_2 - CO_2$) for the trisaccharide with the α2-3 linkage. For the trisaccharide with the α2-6 linkage, two characteristic ion were found at *m/z* 513 ($Y_{1\beta}$) and *m/z* 306 ($^4A_{0\alpha} - CO_2$), this last one also used by other authors[27,28]. Additionally, another ion found at *m/z* 454 ($^{0,2}X_{1\alpha}$ or $^{0,2}X_2$) in both compounds was significantly more intense for the trisaccharide with NeuAcα2-6 linked to GalNAc-ol. In any case, all the mentioned diagnostic ions showed a very low intensity (< 5%), which could be a problem if a differentiation of isomers at low concentrations is required. As it was in our case, Olson *et al.*[29] did not find any of the cited diagnostic ions, even though they analyzed the same compounds using the same type of mass analyzer. Perhaps the low intensity of the diagnostic ions and small differences in the experimental working conditions produced these divergences.

In spite of an exhaustive work on ionization and fragmentation conditions in our triple quadrupole mass spectrometer, the detection of ions at *m/z* 306, 408 or 513 was not accomplished, probably due to their low intensity. Nevertheless, the ion at *m/z* 454 was indeed detected at a low abundance, although this was not very useful since it was present in both isomers (Figs. 1(a) and 1(b)).

Bearing in mind the results obtained so far, we tried to acquire better spectra transferring the analytical method to another mass spectrometer equipped with an ion trap analyzer. Whilst elution conditions remained unchanged, those parameters influencing ionization, desolvatation and ion transmission from source to analyzer were re-optimized for the new analyzer. Strikingly, the mass spectra obtained for both isomers using the ion trap were noticeably different. Thus, the MS^2 spectrum of the trisaccharide with the α2-3 linkage was very similar to that obtained with the triple quadrupole (Fig. 1(c)), and its fragmentation process was in good agreement with that proposed by Domon and Costello[19]. Conversely, the MS^2 spectrum corresponding to the trisaccharide with the α2-6 linkage showed marked differences (Fig. 1(d)). Similar to Robbe *et al.*[26], a small diagnostic ion was observed at *m/z* 513, but the attention was attracted by two more intense ions at *m/z* 615 and 597 (labeled with an asterisk in Fig. 1(d)). In order to ascertain the quality of the spectrum and discard the presence of any interfering compound, blank analysis and high-resolution mass spectra of the precursor ion (*m/z* 675) were performed, without finding isobaric interferences (spectra not shown). The occurrence of artifacts generation during the MS^2 spectrum recording was ruled

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out, as it was registered again after varying several parameters such as time and isolation width of precursor ion, activation q_z value and the amount of precursor ions trapped in the mass analyzer. As a result, no significant differences with the initial MS^2 spectrum were observed, indicating the good quality and reliability of the MS^2 spectra analyses.

Therefore, the next step was to deal with the structural elucidation of the main ions present in the MS^2 spectrum of the Galβ1-3(NeuAcα2-6)GalNAc-ol oligosaccharide. In this sense, the presence of fragments at *m/z* 615 and 597 might be explained by the pathway proposed in Scheme 1a. According to this fragmentation pathway, the ion at *m/z* 615 ($^{2,4}X_{1\beta}$ or $^{1,3}X_{1\beta}$ fragments) appears through the neutral loss of 60 mass units as $C_2H_4O_2$, and the ion at *m/z* 597 by its posterior unspecified dehydration.

In order to confirm this fragmentation mechanism, MS^3 experiments were performed. The ion at *m/z* 675 was, thus, broken and its product ions at *m/z* 615 and 597 were again used as precursors. It was observed that the ion at *m/z* 597 stemmed from ion 615 through the loss of water (MS^3 spectrum not shown), and that the MS^3 spectrum of ion *m/z* 597 presented a rich fragmentation (Fig. 2), in contrast to the scarcely populated MS^2 spectrum obtained from the original precursor (*m/z* 675). Among the resulting fragments, whose identity is discussed below, two facts should be pointed out. First of all, no traces of ions 290 ($B_{1\alpha}$) or 306 ($^4A_{0\alpha} - CO_2$) were detected (see enlarged detail in Fig. 2), allowing us to discard the pathway shown in Scheme 1a. Furthermore, an intense and revealing ion at *m/z* 384 ($Y_{1\alpha}$ or Y_2) was observed, indicating that the fragment lost in the transition 675 > 597 comes from the NeuAc unit. To the best of our knowledge, this process has not yet been described in the literature, and it leads to propose the alternative fragmentation route described in Scheme 1b. This new route is clearly supported by the capability of assigning all the main fragments of the MS^3 spectrum of the ion at *m/z* 597 ($^{4,5}X_{1\alpha} - H_2O$), as it is shown in Fig. 2.

To find an explanation, and taking into account the role that conformation plays in oligosaccharide fragmentation[30], minimum energy conformations were calculated for the ionized oligosaccharides using the MM2 method. Results are shown in Figs. 3(a) and 3(b), where only for the trisaccharide with the α2-6 linkage, the formation of a hydrogen bond between a hydroxyl group of the propanetriol branch sited on the NeuAc unit and the negatively charged oxygen could be observed. Thus, the spatial conformation may justify a proton transfer process between the two groups involved in this hydrogen bond, which would later on induct fragmentation in this area of the molecule, with a neutral loss of $C_2H_4O_2$. The

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viability of this process is supported by a recent work of Zaia *et al*[31], who studied the relevance of proton transfer processes in the negative ion fragmentation of oligosaccharides. On the other hand, it should be noted that the ion at m/z 597 is not observed using beam-type mass analyzers, suggesting that it comes from a slow kinetic process, and agreeing with the fragmentation pathways of Scheme 1b, that involves several rearrangements. To check this point, MS^2 spectra from ion at *m/z* 675 were obtained using activation times ranging from 1 to 1000 ms, and recording the intensities of ions found at *m/z* 597, 454 and 290. These last two ions were selected because are also detected with beam-type mass analyzers, and therefore, generated by a fast kinetic process. Figure 4(a) shows the intensities of ions at *m/z* 597 and 454, relativized to ion at *m/z* 290, against the activation time. As can be seen, the relative intensity of ion at *m/z* 454 remained almost unchanged (fast kinetic), while the ion at *m/z* 597 was very small for collision times below 3 ms, gradually increasing up to 25 ms and showing not significant changes above this value. Thus, this experiment confirm that the ion at *m/z* 597 is generated by a slow kinetic process, compatible with the pathway shown in Scheme 1b, and also justify its absence in mass spectra obtained with beam-type mass analyzers. In fact, a similar behavior has been described for peptides analyzed using an ion trap mass spectrometer[32,33].

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Study of isomeric trisaccharides with NeuGcα2-3 linked to Gal or NeuGcα2-6 linked to GalNAc-ol

With the aim of confirming if this type of fragmentation is characteristic of the preceding oligosaccharides, the same methodology was applied to the following isomers containing NeuGc instead of NeuAc: NeuGcα2-3Galβ1-3GalNAc-ol and Galβ1-3(NeuGcα2-6)GalNAc-ol, and whose structures are displayed in Fig. 5. After the MS optimization process, MS^2 spectra of both oligosaccharides using the triple quadrupole (Figs. 5(a) and 5(b)) and the ion trap (Figs. 5(c) and 5(d)) analyzers were obtained from their respective precursor ions at *m/z* 691 ($[M–H]^-$). As far as we know, this is the first time that these trisaccharides have been detected and characterized by tandem mass spectrometry in their alditol form. Most of the recorded ions were easily assigned through usual fragmentation processes. However, the presence of a very intense ion at *m/z* 597 (60% of the base peak) belonging to the trisaccharide with the α2-6 linkage was observed again, but only in the spectrum obtained with the ion trap (Fig. 5(d)). More surprisingly, the fragmentation of this ion produced a MS^3

spectrum identical to the one shown in Fig. 2. This indicates that this ion (*m/z* 597) is directly derived from a loss in the NeuGc unit and that its structure should be identical to that of the ion at *m/z* 597 ($^{4,5}X_{1\alpha}$ – H_2O) coming from the trisaccharide containing NeuAc. Three-dimensional representations optimized through the MM2 method, and presented in Figs. 3(c) and 3(d), also revealed a hydrogen bond between a hydroxyl group of the propanetriol branch sited on the NeuGc unit and the negatively charged oxygen for the trisaccharide with the α2-6 linkage. As it was the case for NeuAc, such interaction was not found in the trisaccharide with the α2-3 linkage. A tentative fragmentation pathway is shown in Scheme 2, where it is proposed the formation of a cycle as a reaction intermediate, which later on breaks and pursues the described process. The stability and viability of this cycle were also supported by the performed spatial structure calculations.

Likewise, the described process should imply a slow kinetic, which was confirmed with the same type of experiment that was carried out for the oligosaccharide with NeuAc, but using the ion at *m/z* 691 as precursor and recording ions at *m/z* 597, 454 and 306 ($B_{1\alpha}$). It can be seen in Fig. 4(b) that the intensity of ion at *m/z* 454 (relative to ion at *m/z* 306) was almost constant with the activation time, so it should be generated by a fast kinetic process. On the contrary, the ion at *m/z* 597 was very weak below 3 ms and intense only above 25 ms, indicating a slow kinetic process. As it occurred with the NeuAc oligosaccharide, the slow kinetic generation route for ion at *m/z* 597 is compatible with the pathway shown in Scheme 2, and therefore, it would explain the difficulty in its detection by beam-type mass analyzers. The stability and viability of this route have also been supported by the spatial structure calculations carried out, and it is equivalent to the processes found by other authors[31,33].

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Study of NeuGcα2-3Galβ1-3(NeuGcα2-6)GalNAc-ol

Once shown the fragmentation behavior of sialylated trisaccharides, the same type of study was carried out on a tetrasaccharide. The aim of this experiment was to investigate the possible use of fragment at *m/z* 597 as a diagnostic ion, not only for trisaccharides containing sialic acids bound to GalNAc by an α2,6-linkage, but also for longer oligosaccharides. Using the same experimental conditions, the tetrasaccharide NeuGcα2-3Galβ1-3(NeuGcα2-6)GalNAc-ol isolated from equine glycophorines was analyzed by LC-ESI(–)-MS^n with an ion trap mass spectrometer. Its $[M–H]^-$ ion (*m/z* 998) was used as precursor to obtain the MS^2 spectrum shown in Fig. 6(a), where the only significant ion detected corresponds to *m/z* 691,

coming from the lost of a NeuGc unit. As there is one NeuGc unit at both ends of the oligosaccharide chain, it is not possible at this point to know which one is lost. To answer this question, it was necessary to break the ion at *m/z* 691 ($Y_{2\alpha}$ or $Y_{1\beta}$) looking for the corresponding diagnostic ions. The resulting MS^3 spectrum is shown in Fig. 6(b), being obvious an intense ion at *m/z* 597. This ion was subjected to a further fragmentation step, obtaining a MS^4 spectrum identical to the one shown in Fig. 2, which indicates the presence of the Galβ1-3(NeuGcα2-6)GalNAc-ol in the tetrasaccharide. However, the existence of the ion at *m/z* 647 ($[M–H]^- – CO_2$) in the MS^3 spectrum also reveals the presence of the NeuGcα2-3Galβ1-3GalNAc-ol in the tetrasaccharide (see Fig. 5(c)). Therefore, the results indicate that the ion at *m/z* 691 comes from the mixture of the $Y_{2\alpha}$ and $Y_{1\beta}$ fragments.

In short, the tandem mass spectra obtained for this tetrasaccharide reveal that the first breaking process (MS^2) affect the glycosidic bond of NeuGc molecules, generating an ion at *m/z* 691 ($Y_{2\alpha}$ and $Y_{1\beta}$) whose MS^3 spectrum contains characteristics ions from trisaccharides with α2-3 (*m/z* 647) and α2-6 (*m/z* 597) linkages. Consequently, the new diagnostic ion at *m/z* 597 seems to be useful not only to elucidate the structure of sialylated trisaccharides but also for higher sialylated oligosaccharides.

CONCLUSIONS

The results presented in this work clearly show differences among fragmentation processes for α2-6-linked oligosaccharides when ion trap or triple quadrupole analyzers are used. They can be explained bearing in mind how ion traps work, since product ions are not affected by collision voltages, so second and third generation ruptures are not promoted, as it happens with beam-type instruments. Furthermore, they use long collision times, making easier to see slow kinetic reactions, such as molecular rearrangements. These facts have permitted us to describe new fragmentation pathways for the *O*-sialoglycans studied, that do not follow the standard processes described by Domon and Costello[19], and that seem to take relevance only when ion trap instruments are used. For all above, ion traps seem to be more appropriated than triple quadrupoles to develop an analytical method capable of distinguishing between isomeric *O*-linked glycans since a very intense diagnostic ion at *m/z* 597 is detected for molecules containing sialic acids bound to GalNAc by an α2,6-linkage.

Finally, it is recognized that glycans affect the activities of the protein to which they are attached by modulating their functions. Consequently, the developed ion trap multistage mass spectrometric method might be particularly important in the glycobiology field in order to fully understand the potential role of *O*-sialoglycan isomers, as the linkage position of sialic acid groups may involve crucial changes in the biological function of glycoproteins. In this field, advances in mass spectrometry instrumentation are being of great utility for gaining better understanding of the molecular basis of the function of glycoproteins in biological processes.

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FIGURE LEGENDS

Figure 1. Chemical structures and MS^2 spectra of NeuAcα2-3Galβ1-3GalNAc-ol and Galβ1-3(NeuAcα2-6)GalNAc-ol (from bovine CMP) obtained in a triple quadrupole (a and b, respectively) and an ion trap (c and d, respectively) mass analyzers. In all cases, precursor ion was the *m/z* 675.

Figure 2. MS^3 spectrum of the *m/z* 597 fragment ion resulting from MS^2 of *m/z* 675. An enlarged detail of the low *m/z* interval is at the left corner.

Figure 3. Conformations of (a) NeuAcα2-3Galβ1-3GalNAc-ol, (b) Galβ1-3(NeuAcα2-6)GalNAc-ol, (c) NeuGcα2-3Galβ1-3GalNAc-ol and (d) Galβ1-3(NeuGcα2-6)GalNAc-ol. The hydrogen bond between the carbonyl and a hydroxyl group is shown as a dashed line.

Figure 4. Ion ratios obtained for (a) Galβ1-3(NeuAcα2-6)GalNAc-ol, and (b) Galβ1-3(NeuGcα2-6)GalNAc-ol using precursor ions at *m/z* 675 and *m/z* 691, respectively.

Figure 5. Chemical structures and MS^2 spectra of NeuGcα2-3Galβ1-3GalNAc-ol and Galβ1-3(NeuGcα2-6)GalNAc-ol (from equine glycophorines) obtained in a triple quadrupole (a and b, respectively) and an ion trap (c and d, respectively) mass analyzers. In all cases, precursor ion is the *m/z* 691.

Figure 6. Chemical structure, MS^2 (a) and MS^3 (b) spectra of NeuGcα2-3Galβ1-3(NeuGcα2-6)GalNAc-ol (from equine glycophorines) obtained in an ion trap mass analyzer.

Scheme 1. Proposed MS^2 pathways for Galβ1-3(NeuAcα2-6)GalNAc-ol.

Scheme 2. Proposed MS^2 pathway for Galβ1-3(NeuGcα2-6)GalNAc-ol.