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Glycoprofiling of Oligosaccharides of Regular and Lactose-Free Milk by Mass Spectrometry

Umut Şahar[1]*

[1] Department of Biology, Molecular Biology Section, Faculty of Science, Ege University, 35040 Bornova/Izmir, Türkiye
*umut.sahar@ege.edu.tr
*Orcid: 0000-0002-2200-6986

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Abstract

Oligosaccharides from regular milk and lactose-free milk were analyzed by Electrospray Ionization interface coupled with Ion Trap Mass Spectrometry (IT-MS). The negative mode mass spectrometry of sugar compositions was obtained either by direct infusion and mass spectrometry with Liquid Chromatography (LC). Hexose (Hex) and the other oligomeric sugar components were observed in both regular and lactose-free milk. While lactose derivatives (deprotonated lactose dimer, chloride adducts, chloride dimer) in regular milk have seen to be dominated, monosaccharide derivatives (Hex-H_2O, Hex, Hex-Cl) in the lactose-free milk were observed as abundant which was lytic product of lactose. Phosphate/sulfate ester substitution on lactose and sialyllactose in both regular and lactose-free milk samples were observed with similar intensity thus it has been understood that these important milk components are not digested during lactose removing process.

Keywords: Milk, Lactose-free, LC-MS, Oligosaccharides, Sialic acid

1. Introduction

Milk and milk products are valuable food sources because of their high content of essential fatty acids, vitamins, minerals, amino acids, and carbohydrates [1, 2]. Lactose is not present in the milk of some mammals however, it is the main sugar that gives to the energy value of bovine milk [3, 4]. Most mammals show high lactase (β-galactosidase) activity in the infancy period of their lives if milk is their sole food source [3] on the other hand digestion problem of lactose known as lactose intolerance is a common problem in adult human [5] therefore a variety in lactose-free and low-lactose milk or dairy products would be favorable the people who are lactose-intolerant [6]. Industrial production processes and lactose separation from milk by specific methods has been patented [7] and lactose hydrolysis methods and technology have been well reviewed in the literature [8, 9].

Bovine milk glycans are bioactive materials and have vital biological roles comprising the inhibiting of pathogen binding to the gut system and as supplies for beneficial bacteria [10].

Some of studies showing that oligosaccharides may have positive effects on diseases such as diarrhea, necrotizing enterocolitis and allergies related to microbiota [11, 12]. Additionally, it has been attributed that sialylated glycomacropeptide could improve memory and learning in piglets [13]. 3′-sialyllactose (3′-SL) and 6′-sialyllactose (6′-SL) have been shown to possess prebiotic properties that able to reduce stressor-induced alterations and anxiety-like behavior on mice [14]. Considerably glycomics results of milk oligosaccharides of human, bovine, and porcine have been reported which offer opportunities to compare their structural properties [15–17] and it is important to examine the profile and quantity of animal milk oligosaccharides to discover sources of bioactive analogous to human milk glycans [18].

Mass spectrometry has been used extensively both independently and coupled with LC for the investigation and structural characterization of milk oligosaccharides [19–24]. Since the gas phase basicities of some glycans and chloride are close to each other, it has been reported that the chloride adduct ion signal will be higher [25, 26] therefore negative mode analysis would be performed for both neutral and acidic oligosaccharides.

There are numerous of analytical methods used to milk oligosaccharides analysis and in this study it was aimed to characterize the differences or similarities of oligosaccharide in regular and lactose-free milk by mass spectrometry. To the best of my knowledge, there have been no reported applications of MSn and LC-MS/MS for the characterization of regular and lactose-free bovine milk oligosaccharides. It was also aimed to identify sialyllactose and the other sulfate/phosphate substituted lactose in lactose-free milk.

2. Materials and Methods

2.1. Chemicals and Reagents

The organic solvents used in this study were HPLC grade. Acetonitrile was LiChrosolv from Merck (Merck, Darmstadt) and ethanol was from Sigma-Aldrich (USA). The ultrapure water for the mobile phase was obtained using a Sartorius Arium water purification systems (Germany).

2.2. Isolation of Milk Oligosaccharides

Regular and lactose-free milk samples were obtained from local markets. Samples were centrifuged in refrigerated centrifuge at 4 °C for 30 minutes. Following centrifugation upper lipid layer was discarded. The proteins in the remaining sample were then precipitated by adding ethanol in same volume. After precipitation, samples were centrifuged at 4 °C for 30 min again. The supernatant which was containing the oligosaccharides were filtered and then analyzed by mass spectrometry.

2.3. Capillary LC and Ion Trap MS System Parameters

Bruker HCT Ultra ion trap mass spectrometry (Bremen, Germany) was used to perform analyses in negative mode. Data acquisition, processing and the system controls, such as ion transmission voltages, nebulizing gas (Nitrogen) pressure (15.0 psi), dry gas flow (5.0 l/min), and the temperature of dry gas (300 °C) were operated by expert tune mode of Esquire Control software 6.1. Collision and fragmentation of ions operated by Helium in MS analyzer. Multiple-reaction monitoring (MRM) system was used to obtain fragment ion spectra. Total spectral data were collected between m/z 100 and 1500, and scanning were performed in ultrascan mode (26,000 m/z per second). Data Analysis version 3.4 was used for the data processing.

Milk extract was continuously infused at 160 µL/h flow rate using syringe pump to the ESI source. Pseudomolecular ions of monosaccharides and oligosaccharides were both scanned in negative MS and MS/MS mode.

Chromatographic analyses were performed on an Agilent 1200 Series Capillary HPLC system. Separation of sialyllactoses were performed by using ODS column (Zorbax C18 150 × 0.5 mm 5µm). Elution was performed using acetonitrile and water as mobile phase. Chromatographic elution was carried out in gradient mode. Fast reconditioning valve of autosampler was positioned to the direct at the beginning of elution. Sample injection volume was set at 0.1 µl and flow rate of binary pumps were adjusted to 20 µl/min.

3. Results and Discussion

Mass spectrometry has been using as a powerful technique for both structural characterization and quantification of glycans. Mass spectrum for monosaccharides and oligosaccharides mixture from regular milk and lactose-free milk were analyzed by electrospray ionization IT-MS and shown in Figure 1. The samples were obtained from the markets. Milk extracts were scanned by accurate mass and tandem MS options. Some ions in the negative mode corresponded to anionic adducts e.g. $(M+Cl)^-$ and others were $(M-H)^-$ type molecular ions.

3.1. Negative Mode Mass Spectrometry for Oligosaccharides

The negative mode mass spectra profiles in this study provide a crude analysis of sugar compositions of regular and lactose-free milk. Both spectra (Figure 1) were obtained by direct infusion of the solutions of oligosaccharides.extracts.

There are three common disaccharides in nature which were named as sucrose, lactose, and maltose. More than 20 disaccharides which have same molecular weight (m/z 341 in negative mode) have been studied by mass spectrometry [26]. Lactose is a disaccharide (Hex2) also and it is abundant in bovine milk. Although some unknown disaccharides reported in milk [22] pseudomolecular ion m/z 341 was accepted as lactose in this study and m/z 377 likely the chloride adducts of this ion.

The whole mass spectrum profile of regular milk was found to be similar to those discussed and reported previously [27]. In regular milk spectrum that shown in formation of m/z 341. Chloride adducts of lactose at m/z 377 also have intense signal. The other ion m/z 719 correspond to dimer formation of m/z 341 with chloride adducts. Pseudomolecular ions in Figure 1A, m/z 161 was the probable $[M - H]^-$ forms of anhydrohexose (Hex-H_2O), and the other ions 179, 341 and 503 were Hex, Hex2 and Hex3 respectively, as also detected by ESI-MS and comparison with the described data in the literature [28, 29].

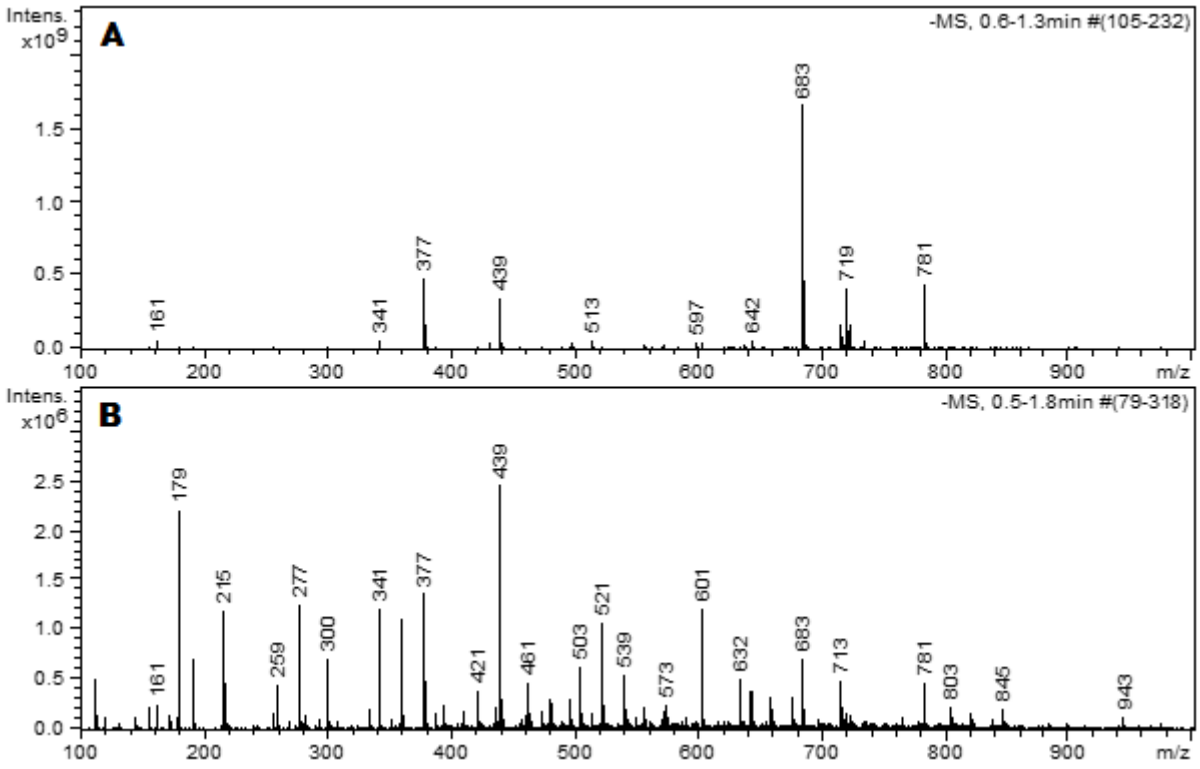

Figure 1. Mass spectrometry results of underivatized oligosaccharides from the milk samples in negative mode by direct infusion. A; Regular Milk, B; Lactose-free Milk.

Anions of m/z 539 was likely the chloride adducts (with isotope of the ^{35}Cl) Hex3 as previously suggested [29] and these ions were observed also in lactose-free milk in Figure 1B.

In the lactose-free milk spectrum (Figure 1B) one of the prominent ion was present corresponding to the composition monosaccharides (Hex) (m/z 179). It was probably the result of the enzymatic digestion reaction of lactose. As similar to the lactose-free milk, greater concentration of galactose and glucose was reported in skimmed milk [22]. m/z 215 reflects the chloride adducts of Hex also that was shown in Figure 1B. Hex (m/z 179) abundance in the regular milk spectrum was lower than the m/z 341 and m/z 377 signal in the Figure 1A. When it was compared to the lactose-free milk the Hex abundance is relatively smaller in regular milk. It was speculated that, m/z 179 was reflects the glucose and galactose which was the digestive product of lactose in lactose-free milk spectrum (Figure 1A).

In the previous studies about glycan ingredients of regular and lactose-free milk, lactose concentration was found to be around 5% (w/v) in regular milk samples and around 0.01% (w/v) in lactose-free labeled milk samples from markets [34-36]. In another study, glucose was found to be 0.05 g/L in regular milk and 19.55 g/L in lactose-free milk [38] which has correlation about results from our study that shown in Figure 1.

3.2. Structural Analysis of Oligosaccharides by Tandem MS

In the literature, m/z 421 in bovine milk was indicated as a phosphorylated dihexose and/or a sulfated dihexose [23, 30]. The fragmentation process of m/z 421 most likely indicate the presence of phosphate/sulfate ester substitution on lactose which was shown in Figure 2 also as reported in literature [24, 27, 29]. A fragment ion at m/z 259 was detected for m/z 439 and 601, which could be evidence of phosphate/sulfate ester substitution in both regular and lactose-free milk (Figure 2). To the best of our knowledge, information on the phosphated/sulfated lactose in milk is rather limited in literature, but important roles on mucous defense mechanism was reported by Ideo et al [42]. Hex3, Hex2HexNAc, 3'-SL and 6'-SL were determined in lactose-free milk that were separated using CarboPac™ PA300-4 µm column [37]. Likewise, we identified phosphate/sulfate ester substituted lactose as additionally.

In this study, more attention has been paid to the analysis of sialic acid-containing glycans especially sialyllactose because it was aimed to observe whether the enzyme used in the production of lactose-free milk digests sialyllactose structure. Sialyllactose has been known an important glycan for microbiota [18]. Mass spectrometry of sialylated oligosaccharides have been

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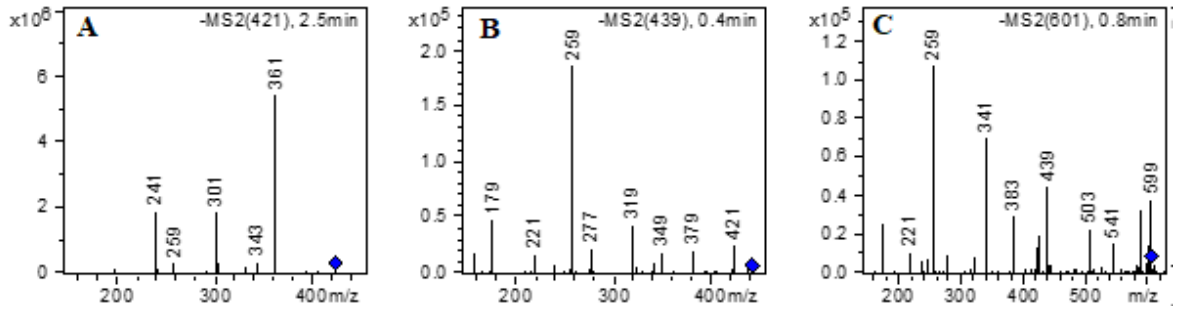

Figure 2. MS/MS analysis of a milk samples. The mass spectrum shows phosphate/sulfate ester substitued oligosaccharide structures and its mass/charge ratio (m/z): A; fragmentation spectrum of m/z 421, B; fragmentation spectrum of m/z 439, C; fragmentation spectrum of m/z 601. Fragment ion at m/z 259 shows phosphate/sulfate ester substitution on oligosaccharides.

widely studied in the previous studies [15, 19, 31]. Sialic acid modification usually occurs on lactose or lactosamine, which results as sialylgalactose, sialyllactose, and sialyllactosamine. The negative mode ESI spectra provide useful information (Figure 3) about sialic acid attached glycans because of the anionic nature of this molecule. Although conventional mass spectrometry is known to yield less information about anomericity and monosaccharide stereoisomers [41], some of fragment ions (Figure 3) in ion trap mass spectrometry that was used in our study provide useful information on linkage positions. Chromatographic separation of glycoforms could be performed by using specific columns and isomeric separation could be done either by state-of-art mass spectrometers. In this study 3'-SL and 6'-SL were not separated on C18 reverse phase column but the results were accepted as satisfactory to claim that 3'-SL and 6'-SL were not digested during lactose removing process.

In the negative mode, m/z 632 corresponds to sialyllactose with the composition 2Hex+1NeuAc. Since these isomers contain the negatively charged sialic acid, it was studied in negative mode by singly deprotonated ions. The IT−MS^2 spectrum of sialyllactoses were shown in Figure 3. In the spectrum the precursor ion $[M-H]^-$ at m/z 632 and the fragment ion at m/z 290 were observed clearly. It was reported that glycoform of the the sialyllactose (α2-3 or α2-6 linkage) have different fragmentation patterns and m/z 306 and m/z 470 look like to be diagnostic ions of the existence of an α2-6-linked sialyllactose (6'-SL), m/z 468 and m/z 408 presence of an α2-3-linked sialyllactose (3'-SL).

3'-SL and 6'-SL are acidic oligosaccharides and they are found in almost all mammalian milk, 3'-SL was reported to be the most abundant bovine milk oligosaccharide [24, 31–33]. Although, 3'-SL and 6'-SL were not separated chromatographically, the Figure 3 shows that 6'-SL is probably abundant form in both regular and lactose-free milk samples because m/z 470 was the prominent ion in the both samples. Low abundance was reported about the Neu5Gc and Fucose-containing oligosaccharides in bovine milk [10] and with this relation these oligosaccharides were not detected in this study.

Milk-based products are applied to several types of nutrients therefore, characterization of the milk samples is very important for the health and food industry. Bovine milk oligosaccharides shows similarity to those found in human milk and characterizing the structures of these sugars is very important for their biological profit [37,39]. In a recent study, more hexosylation (hexose-derived glycation) of amino acids on protein has been detected in lactose-free milk samples [40], with regard to these reults, characterization of glycan components of normal and lactose-free milk may contribute to the glycoproteomic studies.

4. Conclusion

The results obtained in this work provide an insight into the oligosaccharide composition from regular milk and lactose-free milk. From deprotonated and anionic adducts of monosaccharides to larger oligosaccharides were observed and characterized by MS and MS/MS system in this study. Moreover, this study suggests that although lactose intensity in mass spectrum is lower in the lactose-free milk, sialyllactose concentration appears to be closer in both samples and sialyllactoses were not digested in lactose removal process. While lactose adducts were found to be abundant in regular milk, intense hexose adducts were observed in lactose free milk. These findings help to evaluate sugar composition of regular milk and lactose-free milk samples.

Sialoglycans have known to be essential ingredients in human milk and infant formula for infant neural development, construction of microbiota, and defense against pathogenic bacteria. Our results show that both regular and lactose-free bovine milk oligosaccharides could be added to the infant formula, but we must take into account that lactose-free milk contains more glucose which means more "sweet".

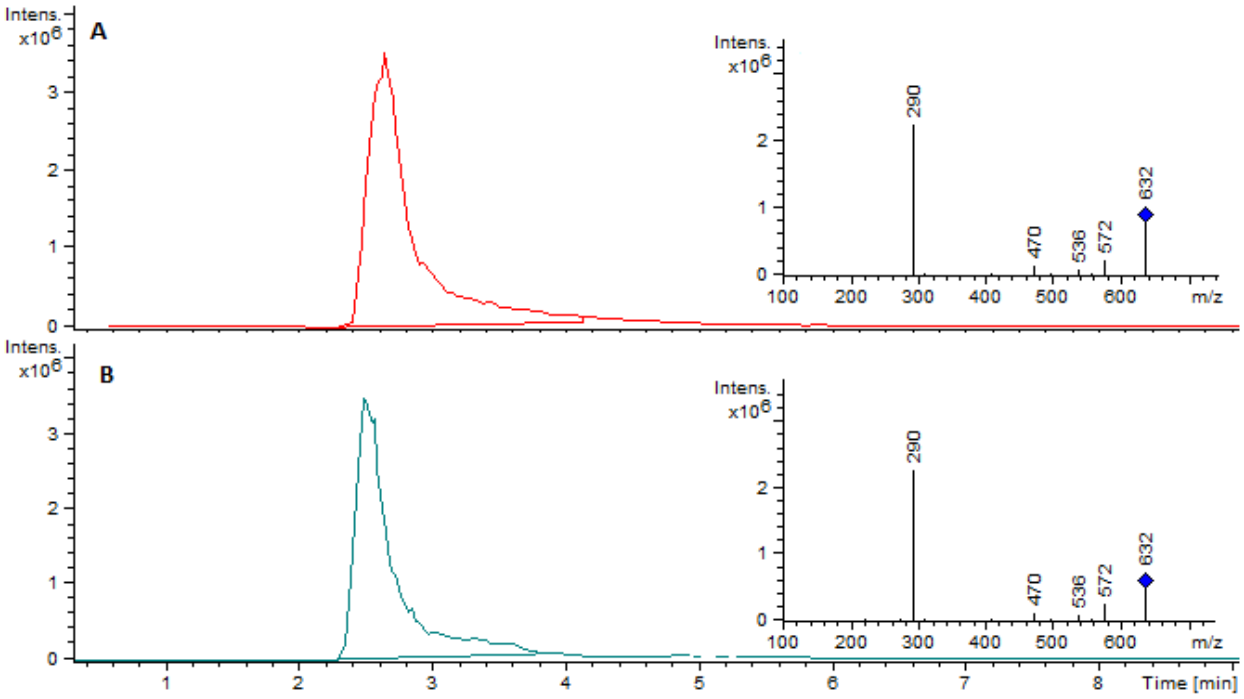

Figure 3. Extracted-ion chromatogram of ion m/z 632. Sialyllactose oligosaccharide (3SL or 6SL) isomers were not separated on the analytical column. A; Sialyllactose in regular milk, B; Sialyllactose in lactose-free milk.

Author's Contributions

Umut Şahar: Drafted and wrote the manuscript, performed the experiment and result analysis.

Ethics

There are no ethical issues after the publication of this manuscript.

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