Chapter 7

Advances in Fractionation and Analysis of Milk Carbohydrates

Veena Nagaraj, Neelam Upadhyay,
Battula Surendra Nath and Ashish Kumar Singh

Additional information is available at the end of the chapter

http://dx.doi.org/10.5772/intechopen.76312

Abstract

Lactose is a principal component of the milk obtained from the mammals. Milk also contains several other sugars such as monosaccharides and oligosaccharides in low concentrations. Lactose has reactive functional groups and can be converted to valuable food-grade derivatives for commercial applications through chemical and/or enzymatic reactions. Physical and chemical properties of carbohydrates determine the methods that can be used for their fractionation and purification. In this chapter, the advanced extraction techniques for fractionation and analytical methodologies applied for the determination of different carbohydrates of milk (lactose, lactulose, and oligosaccharides) are summarized. The main aim of this contribution is to provide the reader with a broad view on the recent fractionation and analytical techniques employed for the analysis of carbohydrates in dairy foods and their applications in food and pharmaceutical industry.

Keywords: lactose, fractionation, purification, analytical techniques, applications

1. Introduction

Lactose is the principal carbohydrate present in milk obtained from all mammalian species, such as cow, buffalo, goat, and sheep. The lactose content in milk is relatively constant, although it varies among different dairy products. Lactose is a disaccharide composed of glucose and galactose molecules linked through β(1–4) glycosidic bond and is synthesized in the mammary gland. Small amounts of free glucose and galactose may also be present [1]. Other minor carbohydrates such as oligosaccharides, glycopeptides, glycoproteins, and nucleotide sugars are also found in milk in very small amounts [2]. The core structures of milk

oligosaccharides have a lactose unit at their reducing end to which specific neutral monosaccharides/oligosaccharides (*N*-acetylglucosamine or *N*-acetylgalactosamine, galactose or glucose, and fucose or 6-deoxyhexose) or acidic oligosaccharides [*N*-acetylneuraminic acid (NANA)] can be attached [3]. However, β-galactooligosaccharides (GOSs) are oligosaccharides composed primarily of galactose and often terminate with a glucose residue at the reducing end, and they occur naturally in the milk of many animals including humans and cows. But, GOSs are normally produced industrially by transgalactosylation of lactose using β-galactosidase.

Substances such as lactulose, lactitol, and lactobionic acid are derived from lactose and are not present in natural sources. When lactose in milk is subjected to moderate heating, its isomerization can occur with the lactulose (4-*O*-β-D-galactopyranosyl-D-fructofuranose) formation through Lobry de Bruyn-Alberda van Ekenstein reaction, through the intermediate compound 1,2-enediol [4]. Consequently, the quantity of lactulose is directly proportional to the intensity of the heat treatment applied and could be useful as indicators of the quality of processing of milk [5]. Lactobionic acid (4-*O*-β-D-galactopyranosyl-D-gluconic acid) is an aldonic acid, comprising of galactose and gluconic acid, obtained through lactose oxidation using a metal catalyst or by enzymatic/microbiological oxidation, while lactitol (4-*O*-β-D-galactopyranosyl-D-glucitol) is a sugar alcohol derived from lactose by catalytic hydrogenation. In this chapter, we describe the different techniques employed for the fractionation of milk carbohydrates, the methods of analysis of fractionated carbohydrates, and applications of fractionated carbohydrates in food and pharmaceutical industry.

2. Need of fractionation of carbohydrates

Intensive biochemical characterization of the carbohydrate molecules and their various bioactivities may facilitate an understanding of their importance in human nutrition and may suggest individual carbohydrate structures to target for industrial production [6]. Recently, many studies reveal the biological significance of some carbohydrates and their potential role as nutraceuticals. However, detailed analysis is beneficial for the identification of the specific oligosaccharides responsible for such activities, as the activity may be attributed to one oligosaccharide or even a fraction of oligosaccharides within the entire milk oligosaccharide pool. Extraction and fractionation techniques represent useful tool in the analysis of such carbohydrates from food samples. Thus, it is important to investigate the structure of oligosaccharides to understand the relationship between their structure and biological function.

3. Techniques employed for fractionation of carbohydrates

The fractionation of carbohydrates from dairy foods is carried out by using some kind of cleanup or extraction methods prior to their analysis. The general scheme for obtaining lactose and other sugars from dairy foods is to first precipitate fat and protein by different reagents (Carrez solution, Biggs-Szijarto solution, and 60% methanol), giving a clear serum adequate for carbohydrate analysis [7]. After precipitation, filtration or centrifugation step yields a clear solution. The ulterior analysis may require an additional step. In this section, the techniques employed for extraction and fractionation of carbohydrates are described.

3.1. Pressurized liquid extraction

Pressurized liquid extraction (PLE) is based on the use of solvents at high temperatures (50–200°C) and pressures (1450–2175 psi) to ensure the rapid extraction rate of compounds [8]. The high temperature enables higher solubility and higher rate of solute diffusion in the solvent, while the application of high pressure maintains the solvent below its boiling point, thereby allowing a high penetration of the solvent into the sample [9]. Recently, the extraction and purification of lactulose from a mixture with lactose have been carried out by using PLE (at 1500 psi) with ethanol/water (70:30, w/w) mixture at 40°C for 30 min, and the recovery of lactulose reached up to 84.4% with a purity of over 90% [10]. Despite the advantages over conventional extraction methods, this method is not found to be suitable for thermo-labile compounds as high temperature can have deleterious effects on their structural and functional activities [11].

3.2. Supercritical fluid extraction

The use of supercritical fluid extraction consists of the separation of the analyte from the matrix using supercritical fluids as the extracting solvent. Carbon dioxide (probably the most used supercritical fluid) is nontoxic, nonflammable, can act at low temperatures, and is relatively cheap; unfortunately, the solubility of carbohydrates in the supercritical phase of this fluid is low [12]. Some of the advantages of supercritical fluid extraction are solvating powers similar to liquid organic solvents, high solute diffusivities, lower viscosity, lower surface tension, and the possibility of adjusting the solvating power by changing pressure or temperature [9]. Carbon dioxide is sometimes modified by co-solvents such as ethanol that change its polarity. This technique is useful for the separation of lactulose and tagatose from their isomeric aldoses (i.e., lactose and galactose, respectively) [13] and GOS from monosaccharides in a commercial sample using CO_2 with ethanol/water as co-solvent (at 150 bar and 80°C) [14].

3.3. Solid phase extraction

Solid phase extraction (SPE) is the very popular technique currently available for rapid and selective sample preparation. The versatility of SPE allows the use of this technique for several purposes, such as purification, trace enrichment, desalting, derivatization, and class fractionation. The selection of an appropriate SPE extraction sorbent depends on understanding the mechanism of interaction between sorbent and analyte of interest [15]. Reverse-phase (RP) cartridges are commonly used for the purification of carbohydrates. Octyl (C_8) and octadecyl (C_{18}) silica phases are the most common RP cartridges used for carbohydrate cleanup. These sorbents show high affinity for hydrophobic compounds but less affinity for hydrophilic solutes such as oligosaccharides [16]. Moreover, C_{18} cartridges are useful for the fractionation of (1–4)-α-glucans depending on their degree of polymerization. Ion-exchange SPEs are used for desalting oligosaccharides mixtures, but care should be taken to avoid the loss of charged sugars during their purification [12]. Solid-phase extraction on graphitized carbon material upon enzymatic amyloglucosidase pre-treatment enabled a good recovery and a selective purification of the different GOS structures from the exceeding amounts of particularly lactose- and maltodextrin-rich preparations [17]. These cartridges are also used effectively to remove salts and residual contaminants (traces of protein and lipids) from whey permeate

samples obtained by ultrafiltration [18] and purified oligosaccharide-rich solutions from bovine colostrums [19], thus allowing proper oligosaccharide identification by mass spectrometry without the need of any further purification.

3.4. Chromatography-based methods

Chromatographic techniques, usually set up in open columns with stationary phases based on anion exchange, adsorption, or gel-filtration/permeation mechanisms, are commonly used for the fractionation of carbohydrates. Brand-Miller et al. [20] used charcoal column chromatography for the separation of human milk oligosaccharides (HMOs) from the other constituents in milk. In this method, milk fat was first removed using centrifugation, and protein precipitated with organic solvents followed by enzymatically converting lactose to glucose and galactose to facilitate separation. The extract was filtered through a column packed with granular charcoal to separate the sugars. Glucose and galactose were eluted from the column initially with water and then with 2% v/v ethanol. The HMOs were then eluted from the column with 50% ethanol. In order to improve detection and characterization of less abundant oligosaccharides from bovine colostrums, fractionation of 2-aminobenzamide-labeled sample into neutral and acidic oligosaccharide fractions was performed by weak anionic exchange chromatography, and its separation ability is based on the combination of the charge and size of molecules [21]. Carbohydrates can be readily fractionated by gel-filtration chromatography on the basis of their relative sizes. To separate the HMO from lactose and salts, gel-filtration chromatography (G25 Sephadex column) has often been used [22–25]. When gel permeation chromatography is used for further separation of the different oligosaccharide fractions, lactose can be obtained separately in one of the fractions [Fractogel TSK HW 40 (S)] [26]. Various problems, however, have limited the development of gel-filtration methods for oligosaccharides. First, many of the commercially available gel-filtration matrices are themselves carbohydrates (e.g., Sephadex, Sepharose, etc.), shedding milligram quantities of heterodisperse carbohydrate polymers into the mobile phase. Second, nonspecific interactions with matrix materials are common because sugars are essentially amphipathic with a hydrophobic ring structure and hydrophilic functional groups [27]. Despite these problems, however, gel-filtration chromatography still remains an important option for the purification of complex oligosaccharides.

3.5. Membrane filtration

Ultrafiltration (UF) and nanofiltration (NF) are increasingly used for the removal of lactose and other soluble components from milk, desalting and separation of interfering compounds; the resulting permeate has numerous applications including the production of lactose. The choice for selecting the UF and NF membrane is mainly based on the value of the molecular weight cutoff (MWCO), which is the molecular mass of the smallest compound retained to an extent larger than 90% [12]. Mehra et al. [28] employed the membrane filtration technology to produce powders enriched in bovine milk oligosaccharides (BMOs) using mother liquor (the liquid remaining after the separation of lactose crystals from whey UF permeate) as a starting raw material. The microfiltrate of mother liquor from the microfiltration step was utilized as the feed to the ultrafiltration (spirally wound membranes with a porosity of 1 kDa MWCO) for fractionation and enrichment of milk oligosaccharides from lactose and mineral salts. NF

is an attractive method for HMO isolation due to the speed with which separations can be performed, and it does not require the use of organic solvents. An easily scalable approach to the recovery of HMO from milk has been developed by Sarney et al. [23], which relay on the combination of enzymatic treatment of defatted and deproteinated milk using β-galactosidase and NF and compared the resulting HMO produced with gel filtration. The authors obtained a yield of 6.7 g of HMO from 1 L of milk in just four NF cycles, yet residual lactose appeared in the oligosaccharide fraction produced with NF but not in that prepared using gel filtration.

4. Methods of analysis of fractionated carbohydrates

4.1. Enzymatic methods

An enzymatic method has been adopted by the IDF [29] for the determination of lactulose content of milk in the presence of much higher concentrations of lactose. Lactulose is often determined by the enzymatic methods using commercially available kits supplied by companies such as Boehringer-Mannheim and Merck. An enzymatic electrochemical method for the detection of lactulose content in milk samples was developed by Moscone et al. [30]. This method uses the enzyme β-galactosidase in solution to hydrolyze lactulose to galactose and fructose, and then the latter is oxidized by a fructose dehydrogenase enzyme reactor using potassium ferricyanide as mediator and platinum-based electrochemical transducer. The sensitivity of the procedure allowed pasteurized, UHT, and in-container sterilized milk can be distinguished. Lactulose content can also be determined by enzymatic method based on amperometric detection [31, 32].

4.2. Spectrophotometric methods

High sensitivity, sufficient accuracy, simplicity, speed, and the necessity of less expensive apparatus make spectrophotometric method as an attractive method for the determination of lactose and lactulose in different dairy products. For the analysis of lactulose preparation, spectrophotometric-enzymatic methods were applied to sugar mixtures produced during isomerization of lactose [33]. A simple spectrophotometric method for lactulose detection was based on hydrolysis of lactulose under acidic conditions followed by reaction with resorcinol, giving absorption peaks at 398 and 480 nm [34]. There are several enzymatic methods based on spectrophotometric detection that have been reported for the determination of lactose or lactulose in milk based on hydrolysis of lactose or lactulose by β-galactosidase [35–37]. A rapid and nondestructive front-face fluorescence spectroscopic method to quantify furosine and lactulose in heat-treated milk has been reported by Kulmyrzaev and Dufour [38]. Zhang et al. [39] developed a sensitive and simple spectrophotometric method for the quantification of lactulose without interference from aldoses. The method was based on hydrolysis of lactulose under acidic conditions. The hydrolyzed product reacted with cysteine hydrochloride-tryptophan reagent, giving an absorption peak at 518 nm.

4.3. Capillary electrophoresis

Capillary electrophoresis (CE) is the technique of choice for the analysis of hydrophilic mono- and oligosaccharides, with an impressive number of different separation approaches and different

detection modes, due to their simplicity, short analysis time, efficiency, and low sample consumption [40, 41]. Bao et al. [42] developed a method for the quantification of sialyl oligosaccharides by CE with detection at 205 nm. However, carbohydrates lack a light-absorbing chromophore, which makes direct UV detection impossible unless a derivatization procedure is involved prior to analysis. Baross et al. [43] determined the lactose in milk and milk products by CE with indirect UV detection. More recently, HMOs have been detected by single CE with laser-induced fluorescence (LIF) [42, 44] or additionally coupled online with MS [45–47]. Labeling of HMO with 8-aminopyrene-1,3,6-trisulfonic acid introduces a fluorophore for the LIF detection, simultaneously adding the negative charge needed for the separation. Albrecht et al. [17] also developed a method for qualitative and quantitative analysis of GOS in different food matrices using CE-LIF. A recent study by Kottler et al. [48] describes the use of multiplexed capillary gel electrophoresis with LIF as a high-throughput method for glycol-analysis, demonstrating the ability to identify and quantify approximately 17 oligosaccharide structures based on "fingerprint" electropherograms.

4.4. Infrared spectrometry methods

Infrared (IR) spectroscopy works by expressing vibrational modes of covalent bonds in molecules and assists in the quantification of any component present in a sample that absorbs IR radiation. The spectral ranges from 750 to 2500 nm and 2500 to 25,000 nm, respectively, are the near infra-red (NIR) and mid-infra-red (MIR) regions. NIR spectra are a result of combination of complex overtones and high frequency, while MIR is due to fundamental stretching, bending, and rotating vibrations in the sample [49]. The use of short-wave NIR wavelengths from 700 to 1100 nm has a key role in the analysis of protein, moisture, fat, and lactose contents in a wide range of dairy products including liquid milk, milk powder, cream, and processed cheese. NIR also offers the possibility of on-line analysis, which avoids the need for batch sampling and minimizes sampling error by averaging of virtually instantaneous, continuous measurements [50]. More recent instrument based on Fourier transform infrared (FTIR) spectroscopy is a rapid, accurate, and nondestructive method that can detect a range of functional groups and is sensitive to change in molecular structure. FTIR provides information on the basis of chemical composition and physical state of the whole sample [51]. Lactulose content in freeze-dried heat-treated milk was determined using diffuse reflectance FTIR spectroscopy in the MIR spectral region of 1286–754 cm^{-1} without any chemical treatment of milk. The authors suggested a broader range of 1300–750 cm^{-1} to be an important region for the study of carbohydrates [52]. FTIR spectroscopy was also used to monitor the lactose and protein concentration, during the UF and NF of whey. A range of 1220–800 cm^{-1} provides details about the lactose content of the sample [53].

4.5. Liquid chromatography

The HPLC is one of the most extensively used techniques employed for the separation of a large variety of carbohydrates in foods [54], as it is particularly advantageous in terms of speed, simplicity of sample preparation (without a prior derivatization), and obtaining a high-resolved chromatogram in a short period of time. A considerable research has been carried out on the quantitative determination of lactose and lactulose by using HPLC. Over all

detectors coupled to HPLC, the refractive index detector (RID) is the most widely used for sugars because no fluorophore (fluorescence detector) or chromophore (UV detector) is necessary; in other words, no derivatization is required. The HPLC-RID was commonly used for the determination of lactose, lactulose, and some other monosaccharides in variety of dairy foods, for instance, milk [55–58], whey permeate [59], cheese [60], and also in some of the milk-based infant formulae [61–63].

The evaporating light scattering detector (ELSD) is a universal, nonspecific detector, which detects all nonvolatile solutes after evaporation of the solvent of the column effluent by light dispersion on the solid analyte particles formed. Recently, Schuster-Wolff-Bühring et al. [64] developed a sensitive and reliable method for simultaneous detection and quantification of lactose and lactulose in milk-based products using HPLC with ELSD and also compared four analytical methods using different HPLC columns. A HPLC column with an amino-bonded polymeric matrix yielded better results compared to amino-bonded silica-phase resin or cation-exchange resin. The detection of nonchromophoric and nonvolatile compounds makes ELSD a suitable technique for the detection of sugars.

High performance (high-pH) anion-exchange chromatography with pulsed amperometric detection (HPAEC-PAD) takes advantage of the affinity between the ionized group of sugars at alkaline pH (pH 12) and a pellicular quaternary amine stationary phase. Therefore, high resolution and highly selective separation of nonderivatized mono-, di- and oligosaccharides can be achieved [65]. This very good match between liquid chromatography and electrochemical detection has allowed the determination of carbohydrates in various complex matrices including dairy foods. Cataldi et al. [66] gave a comprehensive overview of analytical applications in food for carbohydrate analysis by HPAEC-PAD. The sensitive and accurate quantification of lactulose in sterilized milk by HPAEC-PAD has been demonstrated, and it makes the differentiation between sterilized and pasteurized milks possible [67]. The separation of monosaccharides (glucose, galactose, and fructose) and disaccharides (lactose, lactulose, epilactose, sucrose, and maltose) in dairy products [68, 69] and infant formulas [70, 71] by HPAEC-PAD has been described. The quantitative determination of NANA present at the terminal position of many glycoprotein and glycolipid-oligosaccharides by HPAEC-PAD has been reported by Rohrer [72]. Underivatized neutral or acidic oligosaccharides from human milk have been resolved by HPAEC-PAD [73–79]. Sialyllactose isomers (3′- and 6′-sialyllactose) from whey streams were also quantified by HPAEC-PAD [28].

However, since most sugars have no significant UV absorbing and/or fluorophore groups, derivatization prior to separation usually results in higher sensitivity. For detection by UV, a derivatization reagent is required. Reagents such as 1-phenyl-3-methyl-5-pyrazolone (PMP) and p-aminobenzoic ethyl ester are the most popular labels that react with reducing carbohydrates under mild conditions [80, 81]. Common fluorescent tags used for labeling the monosaccharides prior to HPLC analysis are anthranilic acid, 2-aminobenzamide, 2-aminopyridine, phenyl isothiocyanate, 9-fluorenylmethoxycarbonylhydrazine, 7-amino-4-methylcoumarin, and 7-amino-1, 3-naphthalene-disulphonate [82]. Normal-phase [83] and reversed-phase HPLCs [83–92] have been used efficiently for the analysis of derivatized milk oligosaccharides. Retention and separation of the HMO on reversed-phase liquid chromatography depend thus mainly on the method of derivatization; some isomer separation was

obtained so far, but no method has emerged that provides comprehensive isomer separation [93]. Underivatized acidic oligosaccharides or derivatized neutral oligosaccharides have also been resolved by ion-exchange HPLC with detection at 200 nm absorbance [94]. However, the lack of structural discrimination by UV detection can make identification of peaks ambiguous.

Labeling with chromophoric active tags such as 1-phenyl-3-methyl-5-pyrazolone, 2-aminopyridine, and 2-aminobenzoicacid as well as perbenzoylation has been applied for the analysis of oligosaccharides [93]. Recently, hydrophilic interaction liquid chromatography (HILIC)-HPLC has been used for separation and characterization of 2-aminobenzamide-labeled milk oligosaccharides (N- and O-glycans) from bovine colostrum and has proven efficient and robust method for oligosaccharide analysis [21]. Using exoglycosidases, the structures of 37 bovine milk oligosaccharides could be confirmed, which revealed the separation of several structural isomers.

Ultra performance liquid chromatography (UPLC) has also shown improved resolution of *N*-glycans released from glycoproteins [95], with reduced run times and solvent consumption, and it could provide a further improvement in the detection and separation of larger isomeric oligosaccharides.

All the analytical techniques described so far are based on separation alone; however, structural confirmation can in such cases only be obtained based on standards. These standards are expensive and not available for all types of oligosaccharides. For better identification, coupling of the separation with mass spectrometry has proven to be effective. Several applications are reported where sugars were analyzed as their sodium adduct, acetate adduct, or chloride adduct. Indeed, the combination of liquid chromatography and time of flight (TOF) mass spectrometry by the HPLC-chip technology has been extensively and successfully used for oligosaccharide profiling in human milk [96, 97] and bovine milk [19, 98]. Nano-liquid chromatography porous graphitic carbon TOF MS in the positive mode has also been applied in the analysis of HMO [99–101]. Both neutral and sialylated compounds may be separated in one run, and using a library containing retention time, mass, and fragmentation information, immediate identification is possible [100, 101]. Using this method, >200 HMO structures can be separated. More recently, oligosaccharides in bovine colostrum were investigated by Aldredge et al. [102], who employing nano-liquid chromatography tandem mass spectrometry, identified five fucosylated oligosaccharides that were found to be in common with human milk. Nano-LC chip/TOF MS offers an orthogonal dimension of retention time and accurate mass, making it possible to separate isomeric HMOs, with and without sialic acids, in a single-chromatographic separation. Ultra high performance chromatography coupled to mass spectrometry or tandem mass spectrometer (UHPLC-MS or MS/MS) is highly specific and sensitive if single- or multi-reaction monitoring is used. Trani et al. [58] carried out a very interesting comparison of HPLC-RI, LC/MS-MS, and enzymatic assays for the analysis of residual lactose in lactose-free milk. The enzymatic methods as well as HPLC coupled to RI detector are not suitable for quantitative determination of residual lactose in lactose-free milk. But, LC-MS/MS method based on the use of lactose formate adduct was proved to be very sensitive and offered highly reproducible results even at the lowest lactose concentrations.

Porous graphitized carbon (PGC) has also been recognized as a valuable stationary phase for the analysis of native oligosaccharides including neutral [100] and sialylated oligosaccharides [101], mostly because of the extensive separation of isomers that may be obtained. PGC-LC-MS was recently described as the stationary phase of choice for N-glycan analysis [103]. Bao et al. [104] developed a quantitative method for the analysis of HMO using PGC as the stationary phase and single-quadrupole MS detector. This analytical technique provides sensitive, precise, accurate quantification for each of 11 milk oligosaccharides and allows measurement of differences in milk oligosaccharide patterns between individuals and at different stages of lactation.

4.6. Gas chromatography (GC)

Beside LC, GC has also been extensively applied for the determination of carbohydrates in dairy foods. Compared to LC, the main advantage of GC is much higher separation power. However, derivatization is crucial for nonvolatile carbohydrates converted to volatile derivatives amenable to GC analysis. Most of the GC methods are based on the previous trimethylsilylation of sugars. Mainly hexamethyldisilazine, trimethylchlorosilane, *N*,*O*-bis(trimethylsilyl) trifluoroacetamide, trimethylsilylimidazole, and *N*,*O*-bis(trimethylsilyl)acetamide have been used as silylation reagents, and the good volatility and stability characteristics of the derivatives formed make trimethylsilyl (TMS) ethers the most popular derivatives applied to GC analysis of saccharides. Due to the relatively low volatility of carbohydrates, GC analysis is limited to derivatized sugars of low molecular weight, mainly mono-, di-, and trisaccharides [105]. In general, GC methods with flame ionization detection (FID) or MS detection provide a good separation of sugars and a good sensitivity. Recently, the use of MS as detector has increased over the FIDs for the identification of carbohydrates and the determination of molecular mass. Analysis of free carbohydrates in milk and milk products by GC-MS has been described by Reineccius et al. [106]. In this study, free carbohydrates (galactose, glucose, and lactose) in milk products, i.e., milk, cream, and cheeses, were isolated by dialysis, converted to trimethylsilyl ethers, and then analyzed by GC-MS. Troyano et al. [107, 108] developed a GC method for the analysis of free monosaccharides in raw and market milk samples. Valero et al. [109] determined the amount of lactulose formed in pasteurized milk by GC of the TMS derivatives of the free sugar, besides monosaccharides were also determined. Lactulose content was also determined by GC analysis of their corresponding TMS ethers separated on a 50% diphenyl/50% dimethylsiloxane capillary column [110]. Using this method, it was possible to quantify lactulose on different treated milks (UHT, sterilized, pasteurized, condensed, and powder milk). Although GC is a sensitive method for sugar analysis, the sample preparation is laborious and not very practical in routine analysis. Besides in the GC procedure, the anomeric composition of α- and β-anomers is obtained, which means more than one peak area for each compound.

4.7. Mass spectrometry methods

Mass spectrometry provides a sensitive technique to assist in the determination of the structure of carbohydrates, but it is essential that the samples are pure to achieve maximum ionization.

The most widely used ionization methods for oligosaccharides are matrix-assisted laser desorption ionization (MALDI) and electrospray ionization (ESI), coupled with tandem techniques such as collision-induced dissociation (CID). Such advances have improved carbohydrate research with mass spectrometry. The tandem mass spectrometry (MS/MS) has been used for detailed structural analysis of oligosaccharide molecules in which a molecular ion is selected by a first stage of MS, which undergoes activation and fragmentation, and the product ions are analyzed to provide information about the sequence monosaccharide compositions, linkages, and locations of various modifications [111].

Milk oligosaccharides have been analyzed using tandem mass spectrometry such as collision-induced dissociation (CID) and infrared multiphoton dissociation (IRMPD) [112]. A comprehensive label-free procedure to identify and quantify milk oligosaccharides and other glycans using nano-liquid chromatography-time of flight MS was recently developed [113]. During this study, MALDI-Fourier transform ion cyclotron resonance (FTICR) was used for accurate mass measurement compositional analysis. FTICR MS provides exceptional mass accuracy at even few parts per million with extremely high mass resolution and has been extensively and successfully used in complex samples. Oligosaccharides in bovine cheese whey permeate were characterized by a combination of nanoelectrospray (nESI)-FTICR-MS and MALDI-FTICR-MS, and this method was possible to identify 15 (8 were neutral and 7 were acidic) milk oligosaccharides [18]. Recently, Mehra et al. [28] identified 25 oligosaccharides including 6 high molecular weight fucosylated oligosaccharides in a mother liquor of dairy streams using MALDI-FTICR mass spectrometer, and the researchers also confirmed the fucosylated oligosaccharides composition and putative structure by using accurate tandem mass spectrometry.

4.8. Nuclear magnetic resonance (NMR)

Many different analytical techniques have been used to elucidate the free glycan profile present in milk samples. Compared with LC-MS and GC-MS, one of the advantages of NMR spectroscopy is the direct and quantitative relationship between the molar concentration and the intensity of the NMRs. For instance, NMR has played an important role in the structural characterization of free oligosaccharides in human milk [114–117]. Monakhova et al. [118] suggest the use of NMR spectroscopy as screening tool to validate nutrition labeling of lactose-free milk, reporting a detection limit of 30 mg/L.

5. Applications of fractionated carbohydrates

Lactose is used as an agglomerating agent, a flavor enhancer, and a diluent in many foods, beverages, and bakery and confectionery products. In the pharmaceutical industry, it is employed as a diluent in tablets and a carrier in medicines [119, 120]. In the last year, the interest in lactulose has increased to a high extent due to its application in the pharmaceutical and food industry. Lactulose is considered as a prebiotic because it promotes the proliferation of *Bifidobacteria* in the human intestine. Therefore, this disaccharide is used as a food supplement in pediatric and geriatric diets [121] as well as for prevention and treatment of chronic constipation, portal systemic encephalopathy, and other intestinal or hepatical disorders [122]. It has been suggested that lactulose has anti-inflammatory properties, which make it an appropriate and useful adjunctive treatment to inflammatory bowel diseases in humans [123].

Most recently, lactulose has been proposed as a treatment for the *Salmonella* carrier state, a preventative and treatment for high cholesterol, a preventative for gall stones, and an adjunctive treatment for colorectal cancers [124]. Moreover, lactulose is used in pharmaceuticals as a mild laxative and to treat hyperammonemia.

The main characteristics of lactobionic acid include moisturizing, antioxidant, stabilizing, and acidifying capacities. Lactobionic acid appears to be a less commonly used prebiotic for both *Lactobacilli* and *Bifidobacteria*. However, lactobionic acid has been suggested to improve gut health [125]. Other applications of lactobionic acid include as a calcium supplement (calcium lactobionate) in pharmaceutical solutions for stabilizing the organs during transplant [126] and in the cosmetic industry [127]. Lactitol is widely used in chocolates, biscuits, sweets, ice cream, and confectionary manufacture as an alternative to other sugar alcohols, such as mannitol, sorbitol, and xylitol. It is used in low-energy, low-fat, and glycemic foods for diabetics and is also recognized as not causing dental caries [128].

It is widely accepted that milk oligosaccharides play several important protective, physiological, and biological roles including selective growth stimulation for beneficial gut microbiota, inhibition of pathogen adhesion, and immunoregulation [88, 129]. The use of free oligosaccharides and their conjugated derivatives in anti-adhesion therapy may provide an effective therapeutic approach to prevent diseases [21]. GOSs are widely used in infant milk formula, infant foods, follow-on formula, beverages, and fermented milks and in confectionary and bread making, of their functional properties as well as health-enhancing properties. The use of GOS in the livestock feed and a pet food industry is also increasing, especially in the poultry, pig, and aqua-culture [130].

6. Conclusion

There are several fractionations and analytical techniques currently being used for the determination of carbohydrates in different milk and milk products. A problem with studying carbohydrate bioactivities is the limited access to well-defined oligosaccharides. Purification of oligosaccharides from natural sources is laborious, and it is difficult to obtain preparation free of contaminants. In addition, the nonlinear nature of oligosaccharides creates a high structural diversity and their overall complexity makes it difficult for a single analytical method to characterize them. Numerous health benefits of milk oligosaccharides and lactose derivatives are well established, including the pharmaceutical and food applications.

Author details

Veena Nagaraj[1], Neelam Upadhyay[2]*, Battula Surendra Nath[3] and Ashish Kumar Singh[2]

*Address all correspondence to: neelam_2912@yahoo.co.in

1 College of Dairy Science and Technology, GADVASU, Ludhiana, Punjab, India

2 Dairy Technology Division, ICAR-National Dairy Research Institute, Karnal, Haryana, India

3 ICAR-National Dairy Research Institute, Bengaluru, Karnataka, India

References

[1] Haenlein GFW. Goat milk in human nutrition. Small Ruminant Research. 2004;**51**(2): 155-163

[2] Park YW, Júarez M, Ramos M, Haenlein GFW. Physico-chemical characteristics of goat and sheep milk. Small Ruminant Research. 2007;**68**(1-2):88-113

[3] Kunz C, Rudloff S. Health promoting aspects of milk oligosaccharides. International Dairy Journal. 2006;**16**(11):1341-1346

[4] O'Brien J. Non-enzymatic degradation pathways of lactose and their significance in dairy products. In: McSweeney P, Fox PF, editors. Advanced Dairy Chemistry. New York: Springer; 2009. pp. 231-294

[5] Morales FJ, Romero C, Jiménez-Pérez S. Characterization of industrial processed milk by analysis of heat-induced changes. International Journal of Food Science & Technology. 2000;**35**(2):193-200

[6] Ward RE. Isolation of milk oligosaccharides using solid-phase extraction. Open Glycoscience. 2009;**2**:9-15

[7] Egge H, Dell A, Von Nicolai H. Fucose containing oligosaccharides from human milk. Archives of Biochemistry and Biophysics. 1983;**224**(1):235-253

[8] Dunford N, Irmak S, Jonnala R. Pressurised solvent extraction of policosanol from wheat straw, germ and bran. Food Chemistry. 2010;**119**(3):1246-1249

[9] Baiano A. Recovery of biomolecules from food wastes – A review. Molecules. 2014;**19**(9): 14821-14842

[10] Ruiz-Matute AI, Sanz ML, Corzo N, Martín-Álvarez PJ, Ibáñez E, Martínez-Castro I, Olano A. Pressurized liquid extraction with ethanol–water at different temperatures. Journal of Agricultural and Food Chemistry. 2007;**55**(9):3346-3350

[11] Ajila CM, Brar SK, Verma M, Tyagi RD, Godbout S, Valero JR. Extraction and analysis of polyphenols: Recent trends. Critical Reviews in Biotechnology. 2011;**31**(3):227-249

[12] Soria AC, Brok M, Sanz ML, Martínez-Castro I. Sample preparation for the determination of carbohydrates in food and beverages. In: Pawliszyn J, Mondello L, Dugo P, editors. Comprehensive Sampling and Sample Preparation. 1st ed. Oxford, UK: Elsevier, Academic Press; 2012. pp. 213-243

[13] Montañés F, Corzo N, Olano A, Reglero G, Ibáñez E, Fornari T. Selective fractionation of carbohydrate complex mixtures by supercritical fluid extraction with CO_2 and different CO-solvents. The Journal of Supercritical Fluids. 2008;**45**(2):189-194

[14] Montañés F, Olano A, Reglero G, Ibáñez E, Fornari T. Supercritical technology as an alternative to fractionate prebiotic galactooligosaccharides. Separation and Purification Technology. 2009;**66**(2):383-389

[15] Żwir-Ferenc A, Biziuk M. Solid phase extraction technique – Trends, opportunities and applications. Polish Journal of Environmental Studies. 2006;**15**(5):677-690

[16] Packer NH, Lawson MA, Jardine DR, Redmon JW. A general approach to desalting oligosaccharides reseased from glycoproteins. Glycoconjugate Journal. 1998;**15**(8):737-747

[17] Albrecht S, Schols HA, Klarenbeek B, Voragen AGJ, Gruppen H. Introducing capillary electrophoresis with laser-induced fluorescence (CE-LIF) as a potential analysis and quantification tool for galactooligosaccharides extracted from complex food matrices. Journal of Agricultural and Food Chemistry. 2010;**58**(5):2787-2794

[18] Barile D, Tao N, Lebrillab CB, Coisson JD, Arlorio M, Germana JB. Permeate from cheese whey ultrafiltration is a source of milk oligosaccharides. International Dairy Journal. 2009;**19**(9):524-530

[19] Barile D, Marotta M, Chu C, Mehra R, Grimm R, Lebrilla CB, German JB. Neutral and acidic oligosaccharides in Holstein-Friesian colostrum during the first 3 days of lactation measured by high performance liquid chromatography on a microfluidic chip and time-of-flight mass spectrometry. Journal of Dairy Science. 2010;**93**(9):3940-3949

[20] Brand-Miller JC, McVeagh P, McNeil Y, Messer M. Digestion of human milk oligosaccharides by healthy infants evaluated by the lactulose hydrogen breath test. Journal of Pediatrics. 1998;**133**(1):95-98

[21] Mariño K, Lane JA, Abrahams JL, Struwe WB, Harvey DJ, Marotta M, Hickey RM, Rudd PM. Method for milk oligosaccharide profiling by 2-Aminobenzamide Labeling and hydrophilic interaction chromatography. Glycobiology. 2011;**21**(10):1317-1330

[22] Kobata A, Ginsburg V, Tsuda M. Oligosaccharides of human milk. I. Isolation and characterization. Archives of Biochemistry and Biophysics. 1969;**130**(1):509-513

[23] Sarney DB, Hale C, Frankel G, Vulfson EN. A novel approach to the recovery of biologically active oligosaccharides from milk using a combination of enzymatic treatment and nanofiltration. Biotechnology and Bioengineering. 2000;**69**(4):461-467

[24] Jantscher-Krenn E, Zherebtsov M, Nissan C, Goth K, Guner YS, Naidu N, Choudhury B, Grishin AV, Ford HR, Bode L. The human milk oligosaccharide Disialyllacto-N-Tetraose prevents necrotising enterocolitis in neonatal rats. Gut. 2011;**61**(10):1417-1425

[25] Jantscher-Krenn E, Lauwaet T, Bliss LA, Reed SL, Gillin FD, Bode L. Human milk oligosaccharides reduce *Entamoeba histolytica* attachment and cytotoxicity in vitro. The British Journal of Nutrition. 2012;**108**(10):1839-1846

[26] Thurl S, Offermanns J, Muller-Werner B, Sawatzki G. Determination of neutral oligosaccharides fractions from human milk by gel permeation chromatography. Journal of Chromatography B. 1991;**568**(2):291-300

[27] Hodgson J. Chromatogrpahy of complex carbohydrates. Biotechnology. 1991;**9**(2):149-150

[28] Mehra R, Barile D, Marotta M, Lebrilla CB, Chu C, German JB. Novel high-molecular weight fucosylated milk oligosaccharides identified in dairy streams. PLoS One. 2014;**9**(5):e96040

[29] IDF. Provisional Standard. Milk. Determination of Lactulose Content. Enzymatic Method. Standard 175, Brussels: International Dairy Federation; 1995

[30] Moscone D, Bernardo RA, Marconi E, Amine A, Palleschi G. Rapid determination of lactulose in milk by microdialysis and biosensors. Analyst. 1999;**124**(3):325-329

[31] Mayer M, Genrich M, Kunnecke W, Bilitewski U. Automated determination of lactulose in milk using an enzyme reactor and flow analysis with integrated dialysis. AnalyticaChimicaActa. 1996;**324**(1):37-45

[32] Sekine Y, Hall EA. A lactulose sensor based on coupled enzyme reactions with a ring electrode fabricated from Tetrathiafulvalen-Tetracyanoquinodimetane. Biosensors & Bioelectronics. 1998;**13**(9):995-1005

[33] Geier H, Klostermeyer H. Formation of lactulose during heat treatment of milk. Milchwissenschaft. 1983;**38**:475-477

[34] Khan MA, Iqbal Z, Jan MR, Shah J, Ahmad W, Haq ZU. Obaidullah. A spectrophotometric method for quantitative determination of lactulose in pharmaceutical preparations. Journal of Analytical Chemistry. 2006;**61**(1):32-36

[35] Andrews GR. Distinguishing pasteurized, UHT and sterilized milks by their lactulose content. International Journal Dairy Technology. 1984;**37**(3):92-95

[36] Amine A, Moscone D, Palleschi G. Rapid determination of lactulose in milk using Seliwanoff's reaction. Analytical Letters. 2000;**33**(1):125-135

[37] Marconi E, Messia MC, Amine A, et al. Heat-treated milk differentiation by a sensitive lactulose assay. Food Chemistry. 2004;**84**(3):447-450

[38] Kulmyrzaev A, Dufour É. Determination of lactulose and furosine in milk using front-face fluorescence spectroscopy. Le Lait. 2002;**82**(6):725-735

[39] Zhang Z, Wang H, Yang RJ, Jiang XY. A novel spectrophotometric method for quantitative determination of lactulose in food industries. International Journal of Food Science & Technology. 2010;**45**(2):258-264

[40] Wang X, Chen Y. Determination of carbohydrates as their p-sulfophenylhydrazones by capillary zone electrophoresis. Carbohydrate Research. 2001;**332**(2):191-196

[41] Wang X, Wang Q, Chen Y, Han H. Determination of carbohydrates as their 3-aminophthalhydrazide derivatives by capillary zone electrophoresis with on-line chemiluminescence detection. Journal of Chromatography A. 2003;**992**(1-2):181-191

[42] Bao Y, Zhu L, Newburg DS. Simultaneous quantification of sialyloligosaccharides from human milk by capillary electrophoresis. Analytical Biochemistry. 2007;**370**(2):206-214

[43] Baross E, Bolyán D, Ganzler K. Qualitative and quantitative determination of lactose in milk and dairy products by capillary electrophoresis. Periodica Polytechnica Chemical Engineering. 1996;**40**(1-2):117-124

[44] Bao Y, Newburg DS. Capillary electrophoresis of acidic oligosaccharides from human milk. Electrophoresis. 2008;**29**(12):2508-2515

[45] Albrecht S, Schols HA, Van Den Heuvel EG, Voragen AG, Gruppen H. CE-LIF-MS n profiling of oligosaccharides in human milk and feces of breast-fed babies. Electrophoresis. 2010;**31**(7):1264-1273

[46] Albrecht S, Schols HA, Van Den Heuvel EG, Voragen AG, Gruppen H. Occurrence of oligosaccharides in feces of breast-fed babies in their first six months of life and the corresponding breast milk. Carbohydrate Research. 2011;**346**(16):2540-2550

[47] Albrecht S, Schols HA, Van Zoeren D, Van Lingen RA, Groot Jebbink LJ, Van Den Heuvel EG, Voragen AG, Gruppen H. Oligosaccharides in feces of breast- and formula-fed babies. Carbohydrate Research. 2011;**346**(14):2173-2181

[48] Kottler R, Mank M, Hennig R, Müller-Werner B, Stahl B, Reichl U, Rapp E. Development of a high-throughput Glycoanalysis method for the characterization of oligosaccharides in human milk utilizing multiplexed capillary gel electrophoresis with laser-induced fluorescence detection. Electrophoresis. 2013;**34**(16):2323-2336

[49] Upadhyay N, Jaiswal P, Jha SN. Application of attenuated total reflectance Fourier transform infrared spectroscopy (ATReFTIR) in MIR range coupled with chemometrics for detection of pig body fat in pure ghee (heat clarified milk fat). Journal of Molecular Structure. 2018;**1153**:275-281

[50] Osborne B G. Near-infrared spectroscopy in food analysis. In: Meyers RA, editor. Encyclopedia of Analytical Chemistry. USA: John Wiley& Sons, Inc.; 2006. DOI: 10.1002/9780470027318.a1018

[51] Cocchi M, Foca G, Lucisano M, Marchetti A, Paean MA, Tassi L, Ulrici A. Classification of cereal flours by chemometrie analysis of MIR spectra. Journal of Agriculture and Food Chemistry. 2004;**52**(5):1062-1067

[52] Pappas CS, Sakkas L, Moschopoulou E, Moatsou G. Direct determination of lactulose in heat-treated milk using diffuse reflectance infrared Fourier transform spectroscopy and partial least squares regression. International Journal of Dairy Technology. 2015;**68**(3):448-453

[53] Solís-Oba M, Teniza-García O, Rojas-López M, Delgado-Macuil R, Díaz-Reyes J, Ruiz R. Application of infrared spectroscopy to the monitoring of lactose and protein from whey after ultra and nano filtration process. Journal of the Mexican Chemical Society. 2011;**55**(3):190-193

[54] Corzo N, Olano A, Martínez-Castro I. Carbohyrates. In: Nollet LML, Toldra F, editors. Handbook of Dairy Foods Analysis. Boca Raton, FL, USA: Taylor & Francis; 2009. pp. 139-167

[55] Manzi P, Pizzoferrato L. HPLC determination of lactulose in heat treated milk. Food and Bioprocess Technology. 2013;**6**(3):851-857

[56] Silveira MF, Masson LMP, Martins JFP, Álvares TS, Paschoalin VMF, Torre CL, Conte-Junior CA. Simultaneous determination of lactulose and lactose in conserved milk by HPLC-RID. Journal of Chemistry. 2015;**2015**:1-6

[57] Veena N, Nath BS. HPLC determination of polydextrose in fortified milk. Indian Journal of Dairy Science. 2017;**70**(1):123-126

[58] Trani A, Gambacorta G, Loizzo P, Cassone A, Fasciano C, Zambrini AV, Faccia M. Comparison of HPLC-RI, LC/MS-MS and enzymatic assays for the analysis of residual lactose in lactose-free milk. Food Chemistry. 2017;**233**:385-390

[59] Hu M, Kurth MJ, Hsieh YL, Krochta JM. HPLC and NMR study on reduction of sweet whey permeate. Journal of Agricultural and Food Chemistry. 1996;**44**(12):3757-3762

[60] Garde S, Ávila M, Arias R, Gaya P, Nuñez M. Sugars and organic acids in raw and pasteurized milk Manchego cheeses with different degrees of late blowing defect. International Dairy Journal. 2012;**25**(2):87-91

[61] Ferreira IMPLVO, Gomes AMP, Ferreira MA. Determination of sugars and other compounds in infant formulae, follow-up milks and human milk by HPLC-UV/RI. Carbohydrate Polymers. 1998;**37**(3):225-229

[62] Chávez-Servin JL, Castellote AI, López-Sabater MC. Analysis of mono- and disaccharides in milk based formulae by high-performance liquid chromatography with refractive index detection. Journal of Chromatography A. 2004;**1043**(2):211-215

[63] Xinmin W, Ruili Z, Zhihua L, Yuanhong W, Tingfu J. Determination of glucosamine and lactose in milk-based formulae by high-performance liquid chromatography. Journal of Food Composition and Analysis. 2008;**21**:255-258

[64] Schuster-Wolff-Bühring R, Michel R, Hinrichs J. A new liquid chromatography method for the simultaneous and sensitive quantification of lactose and lactulose in milk. Dairy Science & Technology. 2011;**91**(1):27-37

[65] Davidek T, Clety N, Devaud S, Robert F, Blank I. Simultaneous quantification analysis of Maillard reaction precursors and products by high-performance anion exchange chromatography. Journal of Agricultural and Food Chemistry. 2003;**51**(25):7259-7265

[66] Cataldi TRI, Campa C, De Benedetto GE, Fresenius J. Carbohydrate analysis by high-performance anion-exchange chromatography with pulsed amperometric detection: The potential is still growing. Analytical Chemistry. 2000;**368**(8):739-758

[67] Liu Y, Avdalovic N, Pohl C, Matt R, Dhillon H, Kiser R. An on-line, high purity acid and base eluent generation system for ion chromatography. American Laboratory. 1998:48C-54C

[68] Reil JV, Olieman C. Selectivity control in the anion-exchange chromatographic determination of saccharides in dairy products using pulsed amperometric detection. Carbohydrate Research. 1991;**215**(1):39-46

[69] Cataldi TR, Angelotti M, Bufo SA. Method development for the quantitative determination of lactulose in heat-treated milks by HPAEC with pulsed amperometric detection. Analytical Chemistry. 1999;**71**(21):4919-4925

[70] Kaine LA, Wolnik KA. Detection of counterfeit and relabeled infant formulas by high-pH anion-exchange chromatography-pulsed aAmperometric detection for the determination of sugar profiles. Journal of Chromatography A. 1998;**804**(1-2):279-287

[71] Austin S, Bénet T, Michaud J, Cuany D, Rohfritsch P. Determination of *β*-galactooligosaccharides by liquid chromatography. International Journal of Analytical Chemistry. 2014. http://dx.doi.org/10.1155/2014/768406

[72] Rohrer JS. Analyzing sialic acids using high-performance anion-exchange chromatography with pulsed amperometric detection. Analytical Biochemistry. 2000;**283**(1):3-9

[73] Thurl S, Muller-Werner B, Sawatzki G. Quantification of individual oligosaccharide compounds from human milk using high-pH anion exchange chromatography. Analytical Biochemistry. 1996;**235**(2):202-206

[74] Nakhla T, Fu D, Zopf D, Brodsky NL, Hurt H. Neutral oligosaccharide content of preterm human milk. British Journal of Nutrition. 1999;**82**(5):361-367

[75] Coppa G V, Pierani P, Zampini L, Carloni I, Carlucci A, Gabrielli O. Oligosaccharides in human milk during different phases of lactation. Acta Paediatrica Suppliment. 1999;**88**(430):89-94

[76] Finke B, Stahl B, Pfenninger A, Karas M, Daniel H, Sawatzki G. Analysis of high-molecular-weight oligosaccharides from human milk by liquid chromatography and MALDI-MS. Analytical Chemistry. 1999;**71**(17):3755-3762

[77] Bode L, Rudloff S, Kunz C, Strobel S, Klein N. Human milk oligosaccharides reduce platelet-neutrophil complex formation leading to a decrease in neutrophil Beta 2 integrin expression. Journal of Leukocyte Biology. 2004;**76**(4):820-826

[78] Thurl S, Munzert M, Henker J, Boehm G, Muller-Werner B, Jelinek J, Stahl B. Variation of human milk oligosaccharides in relation to milk groups and Lactational periods. The British Journal of Nutrition. 2010;**104**(9):1261-1271

[79] Coppa GV, Gabrielli O, Zampini L, Galeazzi T, Ficcadenti A, Padella L, Santoro L, Soldi S, Carlucci A, Bertino E, Morelli L. Oligosaccharides in 4 different milk groups, Bifidobacteria, and Ruminococcusobeum. Journal of Pediatric Gastroenterology and Nutrition. 2011;**53**(1):80-87

[80] Gomis DB, Tamayo DM, Alonso JM. Determination of monosaccharides in cider by reversed-phase liquid chromatography. AnalyticaChimicaActa. 2001;**436**(1):172-180

[81] Lv Y, Yang X, Zhao Y, Ruan Y, Yang Y, Wang Z. Separation and quantification of component Monosaccharides of the tea polysaccharides from *Gynostemmapentaphyllum* by HPLC with indirect UV detection. Food Chemistry. 2009;**112**(3):742-746

[82] Racaityte K, Kiessig S, Kalman F. Application of capillary zone electrophoresis and reversed phase high-performance liquid chromatography in the biopharmaceutical industry for the quantitative analysis of the monosaccharides released from a highly glycosylated therapeutic protein. Journal of Chromatography A. 2005;**1079**(1-2):354-365

[83] Charlwood J, Tolson D, Dwek M, Camilleri P. A detailed analysis of neutral and acidic carbohydrates in human milk. Analytical Biochemistry. 1999;**273**(2):261-277

[84] Chaturvedi P, Warren CD, Altaye M, Morrow AL, Ruiz-Palacios G, Pickering LK, Newburg DS. Fucosylated human milk oligosaccharides vary between individuals and over the course of lactation. Glycobiology. 2001;**11**(5):365-372

[85] Warren CD, Chaturvedi P, Newburg AR, Oftedal OT, Tilde CD, Newburg DS. Comparison of oligosaccharides in milk specimens from humans and twelve other species. In: Newburg DS, editor. Bioactive Components of Human Milk. Boston, MA: Springer; 2001. pp. 325-332. DOI: 10.1007/978-1-4615-1371-1_40

[86] Martin-Sosa S, Martin MJ, Hueso P. The sialylated fraction of milk oligosaccharides is partially responsible for binding to enterotoxigenic and uropathogenic *Escherichia coli* human strains. The Journal of Nutrition. 2002;**132**(10):3067-3072

[87] Sumiyoshi W, Urashima T, Nakamura T, Arai I, Saito T, Tsumura N, Wang B, Brand-Miller J, Watanabe Y, Kimura K. Determination of each neutral oligosaccharide in the milk of Japanese women during the course of lactation. The British Journal of Nutrition. 2003;**89**(1):61-69

[88] Asakuma S, Akahori M, Kimura K, Watanabe Y, Nakamura T, Tsunemi M, Arai I, Sanai Y, Urashima T. Sialyl oligosaccharides of human colostrum: Changes in concentration during the first three days of lactation. Bioscience, Biotechnology and Biochemistry. 2007;**71**(6):1447-1451

[89] Asakuma S, Urashima T, Akahori M, Obayashi H, Nakamura T, Kimura K, Watanabe Y, Arai I, Sanai Y. Variation of major neutral oligosaccharides levels in human colostrum. European Journal of Clinical Nutrition. 2008;**62**(4):488-494

[90] Leo F, Asakuma S, Nakamura T, Fukuda K, Senda A, Urashima T. Improved determination of milk oligosaccharides using a single derivatization with anthranilic acid and separation by reversed-phase high performance liquid chromatography. Journal of Chromatography A. 2009;**1216**(9):1520-1523

[91] Leo F, Asakuma S, Fukuda K, Senda A, Urashima T. Determination of sialyl and neutral oligosaccharide levels in transition and mature milks of Samoan women, using anthranilic derivatization followed by reverse phase high performance liquid chromatography. Bioscience, Biotechnology and Biochemistry. 2010;**74**(2):298-303

[92] Erich S, Anzmann T, Fischer L. Quantification of lactose using ion-pair RP-HPLC during enzymatic lactose hydrolysis of skim milk. Food Chemistry. 2012;**135**(4):2393-2396

[93] Ruhaak LR, Lebrilla CB. Advances in analysis of human milk oligosaccharides. Advances in Nutrition. 2012;**3**(3):406S-414S

[94] Cardon P, Parente JP, Leroy Y, Montreuil J, Fournet B. Separation of sialyl-oligosaccharides by high-performance liquid chromatography. Application to the analysis of mono-, di-, tri- and tetrasialyl-oligosaccharides obtained by hydrazinolysis of alpha 1-acid glycoprotein. Journal of Chromatography. 1986;**356**(1):135-146

[95] Ahn J, Bones J, Yu YQ, Rudd PM, Gilar M. Separation of 2-aminobenzamide labeled glycans using hydrophilic interaction chromatography columns packed with 1.7 μm sorbent. Journal of Chromatography B. 2010;**878**(3-4):403-408

[96] Ninonuevo MR, Park Y, Yin H, Zhang J, Ward RE, Clowers BH, German JB, Freeman SL, Killeen K, Grimm R, Lebrilla CB. A strategy for annotating the human milk Glycome. Journal of Agricultural and Food Chemistry. 2006;**54**(20):7471-7480

[97] Ninonuevo MR, Ward RE, LoCascio RG, German JB, Freeman SL, Barboza M, Mills DA, Lebrilla CB. Methods for the quantitation of human milk oligosaccharides in bacterial fermentation by mass spectrometry. Analytical Biochemistry. 2007;**361**(1):15-23

[98] Tao N, Depeters EJ, Freeman S, German JB, Grimm R, Lebrilla CB. Bovine milk Glycome. Journal of Dairy Science. 2008;**91**(10):3768-3778

[99] Niñonuevo M, An H, Yin H, Killeen K, Grimm R, Ward R, German B, Lebrilla C. Nanoliquid chromatography-mass spectrometry of oligosaccharides employing graphitized carbon chromatography on microchip with a high-accuracy mass Analyzer. Electrophoresis. 2005;**26**(19):3641-3649

[100] Wu S, Tao N, German JB, Grimm R, Lebrilla CB. Development of anannotated library of neutral human milk oligosaccharides. Journal of Proteome Research. 2010;**9**(8):4138-4151

[101] Wu S, Grimm R, German JB, Lebrilla CB. Annotation and structural analysis of sialylated human milk oligosaccharides. Journal of Proteome Research. 2011;**10**(2):856-868

[102] Aldredge DL, Geronimo MR, Hua S, Nwosu CC, Lebrilla CB, Barile D. Annotation and structural elucidation of bovine milk oligosaccharides and determination of novel fucosylated structures. Glycobiology. 2013;**23**(6):664-676

[103] Jensen PH, Karlsson NG, Kolarich D, Packer NH. Structural analysis of N- and O-glycans released from glycoproteins. Nature Protocols. 2012;**7**(7):1299-1310

[104] Bao Y, Chen C, Newburg DS. Quantification of neutral human milk oligosaccharides by graphitic carbon high-performance liquid chromatography with tandem mass spectrometry. Analytical Biochemistry. 2013;**433**(1):28-35

[105] Ruiz-Matute A I, Hernández-Hernández O, Rodríguez-Sánchez S, Sanz M L, Martínez-Castro I. Derivatization of carbohydrates for GC and GC-MS analyses. Journal of Chromatography B. 2011;**879**(17-18):1226-1240

[106] Reineccius GA, Kavanagh TE, Keeney PG. Identification and quantitation of free neutral carbohydrates in milk products by gas-liquid chromatography and mass spectrometry. Journal of Dairy Science. 1970;**53**(8):1018-1022

[107] Troyano E, Olano A, Fernández-Díaz M, Sanz J, Martínez-Castro I. Gas chromatographic analysis of free monosaccharides in milk. Chromatographia. 1991;**32**(7):379-382

[108] Troyano E, Villamiel M, Olano A, Sanz J, Martinez-Castro I. Monosaccharides and *Myo*-inositol in commercial milks. Journal of Agricultural and Food Chemistry. 1996;**44**(3):815-817

[109] Valero E, Villamiel M, Sanz J, Martínez Castro I. Chemical and sensorial changes in milk pasteurised by microwave and conventional systems during cold storage. Food Chemistry. 2000;**70**(1):77-81

[110] Montilla A, Moreno FJ, Olano A. A reliable gas capillary chromatographic determination of lactulose in dairy samples. Chromatographia. 2005;**62**(5):311-314

[111] An HJ, Lebrilla CB. Structure elucidation of native N- and O-linked glycans by tandem mass spectrometry (tutorial). Mass Spectrometry Reviews. 2011;**30**(4):560-578

[112] Zhang H, Zhang S, Tao G, Zhang Y, Mulloy B, Zhan X, Chai W. Typing of blood-group antigens on neutral oligosaccharides by negative-ion electrospray ionization tandem mass spectrometry. Analytical Chemistry. 2013;**85**(12):5940-5949

[113] De Leoz ML, Wu S, Strum J, Ninonuevo MR, Gaerlan S, Mirmiran M, Ninonuevo M, German JB, Mills DA, Lebrilla CB, Underwood MA. A quantitative and comprehensive method to analyze human milk oligosaccharide structures in the urine and feces of infants. Analytical and Bioanalytical Chemistry. 2013;**405**(12):4089-4105

[114] Martin-Pastor M, Bush CA. Conformational studies of human milk oligosaccharides using ^{1}H-^{13}C one-bond NMR residual dipolar couplings. Biochemistry. 2000;**39**(16):4674-4683

[115] Hu F, Furihata K, Ito-Ishida M, Kaminogawa S, Tanokura M. Nondestructive observation of bovine milk by NMR spectroscopy: Analysis of existing states of compounds and detection of new compounds. Journal Agricultural and Food Chemistry. 2004;**52**(16):4969-4974

[116] Kogelberg H, Piskarev VE, Zhang Y, Lawson A, Chai W. Determination by electrospray mass spectrometry and ^{1}H-NMR spectroscopy of primary structures of variously fucosylated neutral oligosaccharides based on the iso-lacto-n-octaose core. European Journal of Biochemistry. 2004;**271**(6):1172-1186

[117] Chai W, Piskarev VE, Zhang Y, Lawson AM, Kogelberg H. Structural determination of novel lacto-N-decaose and its monofucosylated analogue from human milk by electrospray tandem mass spectrometry and ^{1}H-NMR spectroscopy. Archives of Biochemistry and Biophysics. 2005;**434**(1):116-127

[118] Monakhova YB, Kuballa T, Leitz J, Andlauer C, Lachenmeier DW. NMR spectroscopy as a screening tool to validate nutrition labeling of milk, lactose-free milk, and milk substitutes based on soy and grains. Dairy Science & Technology. 2012;**92**(2):109-120

[119] Seki N, Saito H. Lactose as a source for lactulose and other functional lactose derivatives. International Dairy Journal. 2012;**22**(2):110-135

[120] Majid M, Madadlou A. Maillard conjugation of lactulose with potentially bioactive peptides. Food Chemistry. 2016;**192**:831-836

[121] Schwartz SJ, Sieber FE. The geriatric patient. In: Anesthesia and Uncommon Diseases. 6th ed. Philadelphia: WB Saunders; 2012. pp. 573-585

[122] Corzo-Martínez M, Megías-Pérez R, Montilla A, Olano A., Moreno F J, Villamiel M. Impact of power ultrasound on formation and degradation of lactulose in basic media. EuroCarb. 2013;**17**. Available from: http://hdl.handle.net/10261/115019

[123] Manzanares W, Hardy G. The role of prebiotics and synbiotics in critically ill patients. Current Opinion in Clinical Nutrition and Metabolic Care. 2008;**11**(6):782-789

[124] Bovee-Oudenhoven IM, Termont DS, Heidt PJ, Van der Meer R. Increasing intestinal resistance of rats to the invasive pathogen *Salmonella enteritidis*: Additive effects of dietary lactulose and calcium. Gut. 1997;**40**(4):497-504

[125] Schaafsma G. Lactose and lactose derivatives as bioactive ingredients in human nutrition. International Dairy Journal. 2008;**18**(5):458-465

[126] Saarela M, Hallamaa K, Mattila-Sandholm T, Matt J. The effect of lactose derivatives lactulose, lactitol and lactobionic acid on the functional and technological properties of potentially probiotic *Lactobacillus* strains. International Dairy Journal. 2003;**13**(4):291-302

[127] Alonso S, Rendueles M, Díaz M. Feeding strategies for enhanced lactobionic acid production from whey by *Pseudomonas taetrolens*. Bioresource Technology. 2013;**134**:134-142

[128] Playne MJ, Crittenden RG. Galacto-oligosaccharides and other products derived from lactose. In: McSweeney PLH, Fox PF, editors. Advanced Dairy Chemistry: Lactose, Water, Salts and Minor Constituents. 3rd ed. New York, USA: Springer; 2009. pp. 121-188

[129] Coppa GV, Zampini L, Galeazzi T, Facinelli B, Ferrante L, Capretti R, Orazio G. Human milk oligosaccharides inhibit the adhesion to CaCO-2 cells of diarrheal pathogens: *Escherichia coli*, *Vibrio cholerae*, and *Salmonella fyris*. Pediatric Research. 2006;**59**(3):377-382

[130] Torres DPM, Gonçalves MPF, Teixeira JA, Rodrigues LR. Galacto-oligosaccharides: Production, properties, applications, and significance as prebiotics. Comprehensive Reviews in Food Science and Food Safety. 2010;**9**(5):438-454

IntechOpen