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Characterization of goat colostrum oligosaccharides by nano-liquid chromatography on chip quadrupole time-of-flight mass spectrometry and hydrophilic interaction liquid chromatography-quadrupole mass spectrometry

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ABSTRACT

A detailed qualitative and quantitative characterization of goat colostrum oligosaccharides (GCO) has been carried out for the first time. Defatted and deproteinized colostrum samples, previously treated by size exclusion chromatography (SEC) to remove lactose, were analyzed by nanoflow liquid chromatography–quadrupole-time of flight mass spectrometry (Nano-LC-Chip–Q-TOF MS). Up to 78 oligosaccharides containing hexose, hexosamine, fucose, *N*-acetylneuraminic acid or *N*-glycolylneuraminic acid monomeric units were identified in the samples, some of them detected for the first time in goat colostra. As a second step, a hydrophilic interaction liquid chromatography coupled to mass spectrometry (HILIC-MS) methodology was developed for the separation and quantitation of the main GCO, both acidic and neutral carbohydrates. Among other experimental chromatographic conditions, mobile phase additives and column temperature were evaluated in terms of retention time, resolution, peak width and symmetry of target carbohydrates. Narrow peaks (w_h: 0.2–0.6 min) and good symmetry (A_s: 0.8–1.4) were obtained for GCO using an acetonitrile:water gradient with 0.1% ammonium hydroxide at 40 °C. These conditions were selected to quantify the main oligosaccharides in goat colostrum samples. Values ranging from 140 to 315 mg L^{-1} for neutral oligosaccharides and from 83 to 251 mg L^{-1} for acidic oligosaccharides were found. The combination of both techniques resulted to be useful to achieve a comprehensive characterization of GCO.

1. Introduction

Goat milk is a complex mixture of nutritive and bioactive components with reported health benefits such as carbohydrates, lipids and proteins [1]. Although lactose is the main carbohydrate, presence of other oligosaccharides (OS) similar to those found in human milk, has been reported [2]. Among them, some studies indicate the existence of: (i) neutral oligosaccharides, whose structures are mainly based on lactose with the addition of neutral monosaccharides such as glucose or galactose (Hex), *N*-acetylglucosamine or *N*-acetylgalactosamine (Hex*N*Ac) and fucose or deoxyhexose (Fuc) and (ii) acidic oligosaccharides, containing acidic components such as *N*-acetylneuraminic (Neu5Ac) or *N*-glycolylneuraminic acid (Neu5Gc) [3,4]. Some of these oligosaccharides, such as those containing fucosyl- or sialyl-groups have been described to have prebiotic and pathogen binding activities [5–9]. Although much effort has been focused on the composition, structure and bioactivity of OS in human milk, scarce information about both qualitative and quantitative composition of goat milk OS is available. Since it is well known that bioactive properties are directly related to OS chemical structure, the search of novel sensitive and reproducible methods for the analysis of goat milk OS is of special relevance. Moreover, it is expectable that goat colostrum has higher amounts of OS than goat milk in a similar way to bovine or human milk [3,10], representing an interesting source of bioactive OS.

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Among the different techniques used for OS analysis, high performance liquid chromatography (LC) is one of the most widespread. Human milk OS have been successfully analyzed by normal phase [11] and reverse phase LC [11–13], although a previous derivatization step is required to improve carbohydrates retention [14]. High performance anion exchange chromatography (HPAEC) provides better separation without a previous derivatization step and it has been widely used for goat milk OS characterization and quantitation [2,15–19]. However, the complex profiles obtained for OS mixtures with different linkage variants and the use of high pH and high salts concentrations in mobile phases make this technique not compatible with mass spectrometry (MS), impairing their complete characterization [20].

Hydrophilic interaction liquid chromatography (HILIC) is a powerful LC operation mode for the analysis of complex OS mixtures (galactooligosaccharides, gentiooligosaccharides, etc.), providing an appropriate resolution and good peak shapes [14,20]. Moreover, mobile phases used in HILIC are compatible with MS and even the use of a high percentage of organic solvents enhances the ionization and increase sensitivity which makes this technique appropriate for structural and glycomic research [21]. However, applications of HILIC to the analysis of mammal milks are scarce. Mariño et al. [22] developed a methodology for the analysis of bovine colostrum OS based on their fluorescent labeling, pre-fractionation by weak anionic exchange chromatography and separation by HILIC using an amide based column and a fluorescence detector. Structural assignment of 37 free glycans was carried out by a combination of HILIC analyses, exoglycosidase digestion, desalting and offline MS/MS analyses. HILIC has also been used for the successful determination of six acidic OS in bovine milk, bovine colostrum, and infant formulas [23] in combination with high-resolution selected reaction monitoring mass spectrometry (HILIC-HRSRM-MS). Nevertheless, to the best of our knowledge, HILIC-MS has not been previously used for goat milk OS analysis, being the optimization of the method a requirement for their comprehensive characterization.

In recent years, the use of nano-liquid chip-based technologies mainly coupled to MS or tandem MS (MS/MS) techniques have demonstrated to be extremely helpful for OS identification and it has been applied to milk characterization due to its high sensitivity and capacity for compositional verification [4]. Nano-LC-Chip technology coupled to time of flight (TOF) MS has been successfully used for OS analysis of human milk [24], porcine milk [25] and bovine milk [26,27]. An exhaustive characterization of OS in goat's milks with and without the genetic ability to synthesize α_{s1}-casein by nanoflow liquid chromatography–quadrupole-TOF MS (Nano-LC-Chip–Q-TOF MS) with a porous graphitized carbon column has been recently reported [4]. Twenty nine goat milk OS, 11 of which were detected by the first time, were identified and verified via MS/MS analyses. Moreover, a goat milk oligosaccharide library was also created, which gathered information available in the literature with the new identifications. This methodology has been proven to be an excellent tool for the identification of OS in mammal milks due to its high sensitivity and mass resolution; however, it has not been previously applied to the analysis of goat colostrum samples which could be of interest for further exploitation of goat colostrum oligosaccharides (GCO) as prebiotics.

In this study, goat colostrum samples, previously purified by size exclusion chromatography (SEC) to remove lactose, were firstly submitted to Nano-LC-Chip–Q-TOF MS analysis in order to exhaustively characterize their oligosaccharide fraction. As a second step, a HILIC-MS methodology was developed for the separation and quantitation of the main GCO, both acidic and neutral compounds.

2. Materials and methods

2.1. Chemicals and reagents

All reagents were of analytical grade or better. Acetic acid from Normasolv (Barcelona, Spain), ammonium acetate, ammonium hydroxide from Panreac (Barcelona, Spain) and ethanol of analytical grade were purchased from Lab-Scan (Gliwice, Poland). Acetonitrile (ACN) and formic acid HPLC-MS grade were purchased from Fisher-Scientific (Fair Lawn, NJ, USA). ESI-TOF Low concentration Tuning Mix G1969-85000 was purchased from Agilent Technologies (Santa Clara, CA, USA).

Analytical standards of β-4-galactosyl-lactose, maltotriose and maltotetraose were obtained from Sigma Chemical Co. (St. Louis, MO, USA). 6′-Sialyl-lactose (6′-SL) sodium salt, 3′-sialyl-lactose (3′-SL) sodium salt, 2′-fucosyl-lactose (2′-FL) and 3′-sialyl-*N*-acetyllactosamine were purchased from Carbosynth (Berkshire, UK). Standard solutions in ACN:water (50:50, v:v) were filtered through nylon FH membranes (0.22 μm; Millipore, Bedford, MA, USA) before injection.

2.2. Colostrum samples

For this study, colostrum samples from four Murciano-Granadina goats (CS1–CS4) were obtained from an experimental farm located at Estación Experimental del Zaidín (Granada, Spain). In addition, colostrum from twelve individual Murciano-Granadina goats reared at Hermanos Archiduque farm (Granada, Spain) were collected and pooled (CS5). Collected samples were immediately frozen at −80 °C until further analysis. Animals were cared and handled in accordance with the Spanish guidelines for experimental animal protection (Royal Decree 53/2013 on the protection of animals used for experimentation or other scientific purposes) in line of corresponding European Directive (2010/63/EU). An experimental protocol was approved by the Ethics Committee for Animal Research from the Animal Nutrition Unit.

2.3. Fat and protein removal

Fat and proteins were removed from the samples following the methodology described by Martinez-Ferez et al. [15] with small modifications. Briefly, samples were defatted by centrifugation at 6500 × *g* for 15 min at 5 °C, then kept in an ice bath for 30 min and filtrated through Whatman No. 1 filter paper to remove the supernatant lipid layer, which was discarded.

The total protein fraction was precipitated by adding two volumes of cold ethanol to the skimmed colostrum samples and shaking for 2 h in an ice bath. The solution was then centrifuged at 6500 × *g* for 30 min at 5 °C and supernatant was carefully collected. Ethanol was evaporated from the sample in a rotary evaporator (Büchi Labortechnik AG, Flawil, Switzerland) at 37 °C and the remaining aqueous solution containing the carbohydrate fraction was frozen and lyophilized.

2.4. Colostrum oligosaccharides isolation

Considering the high amounts of lactose present in goat colostrum and the interference of this disaccharide in the analysis of minor oligosaccharides, samples were submitted to SEC fractionation to remove mono- and disaccharides, obtaining an enriched oligosaccharide fraction. Briefly, 25 mL of colostrum carbohydrate solution (20% wt:v) was injected into a Bio-Gel P2 (Bio-Rad, Hercules, CA, USA) column (90 cm × 5 cm) using water as the mobile phase at a flow of 1.5 mL min^{-1} and maintained at 4 °C. The degree of polymerization (DP) of collected fractions was determined by electrospray ionization-mass spectrometry (ESI-MS) on an Agilent