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# On-line high-performance liquid chromatography–fluorescence detection–electrospray ionization–mass spectrometry profiling of human milk oligosaccharides derivatized with 2-aminoacridone

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ABSTRACT

A high-resolution normal-phase high-performance liquid chromatography–fluorescence detection–electrospray ionization–mass spectrometry separation and structural characterization of the main oligosaccharides along with lactose from human milk samples is described. A total of 22 commercially available oligosaccharides were fluorotagged with 2-aminoacridone and separated on an amide column and identified on the basis of their retention times and mass spectra. Derivatized species having mass lower than approximately 800 to 900 exhibited mainly $[M - H]^{-1}$ anions, oligomers with mass up to approximately 1000 to 1100 were represented by both $[M - H]^{-1}$ and $[M - 2H]^{-2}$ anions, and oligomers greater than approximately 1200 to 1300 were characterized by a charge state of −3. Furthermore, the retention times were directly related to the glycans' molecular mass. Human milk samples from the four groups of donors (Se±/Le±) were analyzed for their composition and amount of free oligosaccharides after rapid and simple prepurification and derivatization steps also in the presence of lactose in high content. This analytical approach enabled us to perform the determination of species not detected by traditional techniques, such as sialic acid, as well as of species present in low content easily mistaken with other peaks. Finally, labeled human milk oligosaccharides were analyzed without any interference from excess fluorophore or interference from proteins, peptides, salts, and other impurities normally present in this complex biological fluid.


During the past years, a considerable body of data have confirmed the great benefits of using human milk for healthy term and preterm infants [1,2] due to its well-known nutritional aspects as well as to its biological effects. Oligosaccharides, which are the third most abundant macronutrient component in human milk [2,3], play several important protective, physiological, and biological roles, including growth stimulation for beneficial gut microbiota and inhibition of pathogen adhesion and immunoregulation [4,5]. Oligosaccharides are synthesized in the mammary gland by the action of specific glycosyltransferases through the sequential addition of galactose (Gal),[1] fucose (Fuc), *N*-acetyl-glucosamine (GlcNAc), and sialic acid (NANA) to the lactose molecule [6]. The presence of glycosyltransferases in the mammary gland is genetically determined, with most of them being common to all women with the exception of fucosyltransferases, whose activity is linked to the expression of the Secretor (Se) and Lewis (Le) genes [6,7].

A better understanding of the mechanisms involved in biological processes of oligosaccharides involves a robust characterization of the relationship between glycan structure and functions. Moreover, understanding the structure–activity relationship of the various human milk oligosaccharides may be of value in the design of novel therapeutic agents with anti-infection properties as well as more specific derivatives for artificial nutrition. However, this task has proven to be a challenging one due to the natural complexity of glycans and their unique chemical properties. Considering these aspects, detailed quantitative and qualitative glycan characterization requires the use of specific analytical approaches.

Nuclear magnetic resonance (NMR) has played an important role in the structural characterization of free glycans in milk [8,9]. However, this approach has resulted in a limited application due to the large amounts of sample required and the complexity of the data analysis. High-performance liquid chromatography

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[1] *Abbreviations used:* Gal, galactose; Fuc, fucose; GlcNAc, *N*-acetyl-glucosamine; NANA, sialic acid; Se, Secretor; Le, Lewis; NMR, nuclear magnetic resonance; HPLC, high-performance liquid chromatography; HPAEC–PAD, anion-exchange chromatography–pulsed amperometry detector; UV, ultraviolet; CE, capillary electrophoresis; MS, mass spectrometry; TOF, time-of-flight; AMAC, 2-aminoacridone; MALDI, matrix-assisted laser desorption/ionization; ESI, electrospray ionization; Fd, fluorimetric detector; DMSO, dimethyl sulfoxide; TIC, total ion chromatogram; HILIC, hydrophilic interaction liquid chromatography; MS/MS, tandem mass spectrometry.

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(HPLC) has been extensively applied to milk oligosaccharides resolved by anion-exchange chromatography and detected by pulsed amperometry (HPAEC–PAD) [2,10] or by ion-exchange HPLC and ultraviolet (UV) detection [11]. However, even if these techniques have proven to be efficient and simple, most glycans have no significant UV absorption, and derivatization with fluorophore groups prior to separation usually results in higher sensitivity. As a consequence, analysis of derivatized milk oligosaccharides by HPLC [3,12] and capillary electrophoresis (CE) [13] has been developed. However, UV or fluorescence detection does not provide specific structural information, and this is a key point also related to the highly complex nature of the milk glycan mixtures. Furthermore, these analyses require the use of standards and further separation runs to calibrate the system. As a consequence, for a specific determination of complex mixtures of oligosaccharides, analytical separation is usually required with evaluation by means of mass spectrometry (MS). In fact, during recent years, MS analysis has proven to be extremely beneficial in the field of glycan identification and has been successfully applied to milk oligosaccharides, resulting in compositional analysis [8,9,12,14]. Finally, the combination of liquid chromatography and time-of-flight (TOF) MS by the HPLC–chip technology has been extensively and successfully used for oligosaccharide profiling in various milk samples [15,16]. By using these modern analytical methods, several hundreds of human milk oligosaccharides have been identified [17,18]. However, it is important to underline that the main quantitative component of human milk glycans is made up of 20 to 25 oligosaccharides, with the remaining ones constituting only minimal amounts. As a consequence, robust sensitive and specific analytical separation to profile these main oligosaccharide species would be of interest for a rapid screening of several kinds of milk samples also in connection with their variation related to secretory groups and day of lactation [2].

2-Aminoacridone (AMAC) is a well-known fluorescent hydrophobic molecule that has been successfully used for the derivatization and separation of various complex oligo(di)saccharides [12,19–21]. However, to date, only one article has reported the separation of AMAC-derivatized human milk oligosaccharide with off-line matrix-assisted laser desorption/ionization (MALDI)–TOF and electrospray ionization (ESI) characterization [12]. In fact, hydrolyzed dextran components were necessary to obtain glucose equivalent value and oligosaccharide size information. On the contrary, here we report an on-line HPLC–ESI–MS separation and structural characterization of more than the 20 principal human milk oligosaccharides derivatized with AMAC, providing a high-resolution system also applicable by using on-line an even more sensitive fluorimetric detector (Fd) in addition to ESI–MS. Furthermore, oligosaccharides were separated and detected along with the simultaneous presence of lactose also derivatized and quantitatively evaluated with no previous specific elimination phase. The method described here was applied to the determination of oligosaccharides in the four secretory groups and quantitatively compared with the common HPAEC–PAD separation [2].

## Materials and methods

### *Materials and reagents*

Various milk oligosaccharides (see Table 1) were obtained from Sigma–Aldrich. AMAC (>98%), glacial acetic acid, dimethyl sulfoxide (DMSO, 99.9%), sodium cyanoborohydride (95%), acetonitrile (MS grade), and all other reagents of the purest grade available were obtained from Sigma–Aldrich.

Single human milk samples from mothers of group 1 (Se+/Le+), group 2 (Se−/Le+), group 3 (Se+/Le−), and Group 4 (Se−/Le−) were obtained from the Pediatric Division, Department of Clinical Sciences, Polytechnic University of the Marche (Ancona, Italy). Human milk samples were obtained from healthy mothers having delivered term newborns during the first month of lactation.

### *Extraction of oligosaccharides from human milk samples*

Acetonitrile (2 ml) was added to 2 ml of human milk samples. After centrifugation at 5000 rpm for 20 min, supernatants were filtered on 0.2-μm filters and lyophilized. Dried material was derivatized with AMAC as reported below.

### *Derivatization of oligosaccharides with AMAC*

Derivatization of various glycan standards or glycans from different human milk samples with AMAC was performed as described previously [20,21] with minor modifications. Pure glycans or lactose (100 ng) were reconstituted with 5 μl of a 0.1-M AMAC solution in glacial acetic acid–DMSO (3:17, v/v) and 5 μl of a freshly prepared solution of 1 M sodium cyanoborohydride in water. Then, the mixtures were centrifuged in a microfuge at 11,000*g* for 3 min. Derivatization was performed by incubating at 45 °C for 4 h. Finally, 15 μl of 50% (v/v) DMSO was added to the samples, and aliquots were taken for HPLC–Fd–ESI–MS analysis.

Oligosaccharides extracted from 2 ml of various human milk samples were reconstituted with 10 μl of a 0.1-M AMAC solution in glacial acetic acid–DMSO (3:17, v/v) and 10 μl of a freshly prepared solution of 1 M sodium cyanoborohydride. After centrifugation, derivatization was performed by incubating at 45 °C for 4 h. Then, 30 μl of 50% (v/v) DMSO was added to the samples and 20 μl was injected in HPLC–Fd–ESI–MS.

### *HPLC–Fd–ESI–MS*

HPLC separation was performed on a 3.5-μm XBridge amide column (250 × 4.6 mm) obtained from Waters. Eluent A was acetonitrile, and eluent B was 200 mM ammonium formate (pH 4.5). After sample injection, an isocratic separation was performed with 25% eluent B in 60 min. After this, gradient from 25% to 35% eluent B in 90 min and from 35% to 90% B in a further 20 min, at a flow rate of 0.5 ml/min, was used. When applied, the Fd from Jasco (model FP-1520), with an excitation wavelength of 428 nm and an emission wavelength of 525 nm, was connected on-line before the ESI–MS equipment. The column effluent entered the source of the ESI–MS for continuous detection by MS. ESI mass spectra were obtained using an Agilent 1100 VL series (Agilent Technologies). The electrospray interface was set in negative ionization mode with the capillary voltage at 3500 V and a heat source of 350 °C in scan spectra from 250 to 1500 Da (10 full scans/s) with a maximum accumulation time of 300 ms and an ICC target of 20,000. Nitrogen was used as a drying gas (12 L/min) and a nebulizing gas (70 psi). Skimmer 1 was used at −40 V to acquire spectra having maximum intensity. Accordingly, the capillary exit offset was −117.2 V. The trap drive was 42.0. Software versions were 4.0 LC/MSD Trap Control 4.2 and Data Analysis 2.2 (Agilent Technologies). The oligosaccharides were quantified using the peak area of interest determined by retention time and *m*/*z* values from the total ion chromatogram (TIC).

## Results and discussion

### *Oligosaccharide standards*

Fig. 1A shows the TIC obtained for the 21 commercially available oligosaccharides derivatized with AMAC along with underivatized NANA (species 16). The separation and detection of the