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Validation and application of a method for the simultaneous absolute quantification of 16 neutral and acidic human milk oligosaccharides by graphitized carbon liquid chromatography – electrospray ionization – mass spectrometry

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Lacto-*N*-difucohexaose I (PubChem CID: 3082109)
Lacto-*N*-difucohexaose II (PubChem CID: 53262301)
Lacto-*N*-tetraose (PubChem CID: 440993)
Lacto-*N*-hexaose (PubChem CID: 194173)
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ABSTRACT

Human milk oligosaccharides (HMOs) are free glycans naturally present in human milk that act as prebiotics, prevent pathogen binding, modulate the immune system and support brain development in infants. The HMOs composition and concentrations vary significantly among different women mainly because of the direct influence of the Secretor and Lewis phenotypes on HMOs biosynthesis. Analytical methods that can identify the differences in the HMOs composition and concentrations are a fundamental tool in HMOs research. This paper describes a simple HMOs extraction and analysis for the simultaneous and absolute quantification of neutral and acidic HMOs by graphitized carbon liquid chromatography–electrospray ionization–mass spectrometry. This method was validated and applied to analyze HMOs in the human milk obtained from 10 women. This method allows accurate and reliable quantification of HMOs and can be used to determine differences in HMOs concentrations throughout lactation and among women with different Secretor and Lewis phenotypes.

1. Introduction

Human milk oligosaccharides (HMOs) are free glycans composed of 3–22 monosaccharide units and constitute the third most abundant solid component of human milk, after lactose and lipids (Newburg & Neubauer, 1995; Ninonuevo et al., 2006). The total HMOs concentration is approximately 20–23 g/L in colostrum and 5–15 g/L in mature human milk (Coppa et al., 1999; Urashima & Taufik, 2010). The HMOs fraction is highly complex, containing at least 200 distinct structures, of which approximately 100 have been identified (Ninonuevo et al., 2006). However, approximately 20 HMOs constitute the major part (~80%) of the total HMOs fraction, and the remaining ones are present in low concentrations (Ninonuevo et al., 2006; Zivkovic, German, Lebrilla, & Mills, 2011). Despite their high concentration and diversity,

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and unlike the other major nutrients in human milk, HMOs are not digested by the infant and reach the large intestine in its intact form, where they are metabolized by certain bacteria, especially of the genus *Bifidobacterium*, or excreted intact in the feces (Chaturvedi, Warren, Buescher, Pickering, & Newburg, 2001; Coppa et al., 2001; De Leoz et al., 2015).

HMOs consist of the monosaccharides glucose (Glc), galactose (Gal), *N*-acetylglucosamine (GlcNAc), fucose (Fuc) and sialic acid, in the form of *N*-acetylneuraminic acid (Neu5Ac) (Bode & Jantscher-Krenn, 2012). However, HMOs do not always contain all 5 monosaccharides in their composition. Depending on the monosaccharides present in the structure, HMOs can be classified into three main groups: *1. neutral core HMOs*, which contain only Glc, Gal and GlcNAc; *2. neutral fucosylated HMOs*, which contain one or more Fuc units; and *3. acidic HMOs*, which contain one or more Neu5Ac units.

HMOs biosynthesis occurs in the mammary gland starting from a lactose molecule that can be elongated by the addition of GlcNAc and Gal to form a neutral core structure, which may or may not involve the addition of Fuc and Neu5Ac. Fuc can be linked to the HMO chain by three different linkages (α1-2, α1-3 and α1-4) but can also be directly attached to lactose through α1-2 or α1-3 linkages. Neu5Ac can be linked to the HMO chain or linked directly to lactose through α2-3 or α2-6 linkages (Chen, 2015). The Fuc addition is directly influenced by the activity of the Secretor (*Se*) and Lewis (*Le*) genes in the woman. The *Se* gene encodes the enzyme α1-2 fucosyltransferase (FUT 2), which is responsible for linking Fuc to the HMO through an α1-2 linkage. The *Le* gene encodes the enzyme α1-3/4 fucosyltransferase (FUT 3), which attaches Fuc to the HMO through an α1-4 linkage. Women with mutations in the *Se* and *Le* genes lack the FUT 2 and FUT 3 enzymes and consequently do not produce α1-2 and α1-4 fucosylated HMOs, respectively. According to the activity of the *Se* and *Le* genes in a lactating woman, HMOs composition can be classified into four groups: *1.* $Se+Le+$, the most common group, containing all fucosylated HMOs with α1-2, α1-3 and α1-4 linkages, such as 2′-FL, 3′-FL and LNDFH I; *2.* $Se-Le+$, which does not contain α1-2 fucosylated HMOs; *3.* $Se+Le-$, which does not contain α1-4 fucosylated HMOs; and *4.* $Se-Le-$, the least common phenotype, which does not contain α1-2 and α1-4 fucosylated HMOs (Thurl, Henker, Siegel, Tovar, & Sawatzki, 1997). However, these four groups contain α1-3 fucosylated HMOs, such as 3′-FL and DFpLNnH, whose synthesis apparently is not influenced by the *Se* and *Le* genes. In addition to the differences in the types of HMOs produced, the activity of the *Se* and *Le* genes also significantly affects the individual and total HMO concentrations in human milk (Thurl et al., 2010). Thus, the HMOs composition and concentrations present significant differences in human milk from different women.

The great structural diversity of the HMOs results in these molecules having a broad range of biological functions, such as promoting a healthy intestinal microbiota, inhibiting the adhesion of pathogens to the surface of the host cell, modulating the immune system, promoting postnatal brain development and providing natural protection against necrotizing enterocolitis (Bode, 2012). Several studies have demonstrated that the biological functions of HMOs are structure specific. For example, regarding the prebiotic effects of HMOs, some studies have suggested that certain HMOs are preferentially consumed by commensal bacteria (LoCascio et al., 2007). Although it can utilize any of the HMOs, *Bifidobacterium longum* subsp. *infantis* prefers short-chain HMOs containing up to 7 monosaccharide units (Asakuma et al., 2011). A significant reduction in the amount of intact lacto-*N*-tetraose, lacto-*N*-neotetraose, 2′-FL and acidic HMOs excreted in infant feces occurs concomitantly with an increase in the *Bifidobacterium* population, suggesting that these HMOs were preferentially consumed by *Bifidobacterium* in the infant intestine (Davis et al., 2016). To identify the HMOs performing specific functions and at what concentration they exert their effects, as well as to investigate whether different HMOs compositions and concentrations in human milk lead to distinct outcomes in the infant, accurate and reliable methods for the identification and exact quantification of HMOs are a fundamental tool.

The most commonly used methods for the absolute quantification of HMOs have thus far been based on HMOs separation by high-performance liquid chromatography (HPLC) coupled to ultraviolet (UV), fluorescence (FD) or pulsed amperometric (PAD) detection (Asakuma et al., 2008; McGuire et al., 2017; Musumeci, Simpore, D'Agata, Sotgiu, & Musumeci, 2006). Capillary electrophoresis (CE) with either UV or FD detection is another option for HMO analysis (Galeotti et al., 2014; Olivares et al., 2015). However, to provide accurate HMOs quantification, these methods usually require a large sample volume (> 1 mL) and time-consuming sample preparation, in most cases involving the derivatization or fractionation of the HMO extract into neutral and acidic HMOs prior to analysis. In addition, these methods are not structurally selective, as they are based on separation alone, and separation is not always achieved due to the high degree of complexity and isomerism of the HMOs mixture. To overcome this problem, coupling the separation technique with mass spectrometry (MS) has proved to be successful for HMOs identification (Bao, Chen, & Newburg, 2013; Oursel, Cholet, Junot, & Fenaille, 2017). Due to the ability of porous graphitic carbon (PGC) to resolve isomeric structures, combining the use of PGC columns with MS is an increasingly common method for profiling and quantifying HMOs in human milk samples and has become a powerful tool in HMO analysis (Bao et al., 2013; Hong et al., 2014; Ninonuevo et al., 2006; Oursel et al., 2017; Ruhaak & Lebrilla, 2012).

However, there is currently a small number of methods by liquid chromatography coupled to mass spectrometry (LC–MS) available for HMOs analysis. In a recent systematic review of oligosaccharides concentrations in human milk, only 3 of 21 studies used LC–MS to measure HMOs concentrations (Thurl, Munzert, Boehm, Matthews, & Stahl, 2017). One of these studies was conducted by Bao et al. (2013), which presented a thoroughly validated LC–MS method for the absolute quantification of HMOs. However, it was developed and validated only for neutral HMOs, not incorporating important acidic HMOs such as 6′-sialyl-lactose (6′-SL) and 3′-sialyl-lactose (3′-SL). The other 2 studies used methods for absolute quantification of neutral and acidic HMOs, although for a small number of individual HMOs. Goehring, Kennedy, Prieto, & Buck (2014) reported absolute concentrations for 2′-FL and 6′-SL, and Hong et al. (2014) used a method for absolute quantification of 7 HMOs: 4 neutral and 3 acidic. A study using LC–MS not included in the systematic review from Thurl et al. (2017), was published by Xu et al. (2017), in which a pooled human milk sample was used as standard for the construction of the calibration curve and concentrations were reported for total HMOs, total neutral core HMOs, total neutral fucosylated HMOs, total acidic HMOs and groups of HMOs having the same monosaccharides composition (structural isomers). The concentrations of individual HMOs were measured only for the fucosylated 2′-FL, 3′-FL and lacto-difucotetraose (Xu et al., 2017). Therefore, data on absolute concentrations of various individual HMOs are scarce and there is a need for sensitive and reliable methods for the accurate quantification of individual HMOs.

The aim of this work is to develop and validate a reliable method for the absolute quantification of the most abundant oligosaccharides in human milk, including representative neutral core, neutral fucosylated and acidic HMOs in a single analysis. In this paper, we describe a validated method for the identification and absolute quantification of 4 neutral core, 7 neutral fucosylated and 5 acidic HMOs in human milk, which are extracted and analyzed simultaneously in the same chromatographic run. The validated method was applied to analyze the HMOs composition and concentrations in real human milk samples obtained from 10 women. The method allows accurate and reliable quantification of HMOs and can be used to characterize human milk samples in terms of the *SeLe* phenotype, as well to verify changes in HMOs composition and concentrations throughout lactation stages.