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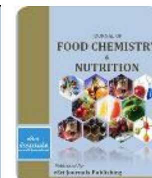

A VALIDATED METHOD FOR DETECTION AND QUANTITATION OF 2'FUCOSYLLACTOSE IN INFANT FORMULA MATRICES

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A B S T R A C T

Analytical methods to assess the content of free carbohydrates in solution range from simple tests of reductive power to combinations of chromatography and mass spectrometry. Soluble carbohydrates such as lactose, maltose, fructooligosaccharides, and galactooligosaccharides are commonly found in infant formulas either as sources of energy or soluble fibers. On the other hand, a rich repertoire of lactose-based carbohydrates occurs naturally in human milk. The advent of novel biosynthesis technologies resulted in the availability of human milk oligosaccharide structures that are being used as ingredients in infant formulas. Notably, 2'Fucosyllactose has been tested in preclinical and clinical studies to determine its safety and to explore its potential health benefits in the context of pediatric nutrition. Several chromatographic methods for the analysis of human milk oligosaccharides have been published and, the main challenge associated with 2'Fucosyllactose quantitation has been to improve its resolution from lactose, which is present at concentrations around 70 g/l in both, infant formula and human milk. We developed a high-performance anion exchange chromatography method to detect and quantify 2' Fucosyllactose in the presence of lactose by expanding the elution time between these saccharides. We validated the analytical procedure which behaved linearly (average R=0.99951) at concentrations as low as 1.75 µg/ml (limit of quantitation) with an average limit of detection of 0.43 µg/ml.

Keywords: 2'Fucosyllactose, oligosaccharides, infant formula, lactose, HPAEC, HMO, human milk.

INTRODUCTION

Human milk oligosaccharides (HMO) have long been studied by researchers seeking to elucidate their biological functions (Plaza-Diaz *et al.*, 2018; Ramani *et al.*, 2018; Donovan and Comstock 2016; Vazquez et al., 2015; Kunz, 2012). Among the neutral (not acidic) HMO, 2'Fucosyllactose (2'FL) has attained prominence as a subject of research due to its addition to infant formula (Austin *et al.*, 2018; Vandenplas et al., 2018) and the discovery of its presence in the plasma of breastfed infants (Goehring *et al.*, 2014; Ruhaak *et al.*, 2014). While mass spectrometry is frequently used to characterize carbohydrate mixtures (Pfenninger *et al.*, 2008; German *et al.*, 2008), the most favored analytical approach to resolve oligosaccharides in industrial settings is High-Performance Anion Exchange Chromatography (HPAEC) with Pulsed Amperometric Detection (PAD). Methods based on HPAEC-PAD have been used to analyze HMO in samples from collections of human milk specimens (Castanys *et al.*, 2013; Thurl *et al.*, 2010; Erney *et al.*, 2000), non-human milk samples (Mudd *et al.*, 2016; Prieto 2012) and infant formulas (Austin *et al.*, 2018; Prieto 2005). 2'FL is synthesized in the lactating mammary gland by the action of a fucosyltransferase that is genetically determined (Castanys *et al.*, 2013). Around 80% of women do not express 2'FL in their milk since they lack the gene that encodes a functional synthetic enzyme. The variability of glycoconjugates of human milk from sample to sample is highlighted by the absence or presence of 2'FL and other fucosylated oligosaccharides that depend on genetically determined glycosyltransferases for their synthesis (Erney *et al.*, 2000). When present in human milk, 2'FL tends to be the most abundant HMO and normally elutes either near to

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lactose or as a shoulder of the lactose peak in most chromatography-based protocols. With the advent of technologies to synthesize HMO (Huamng *et al*., 2017) the need to separate 2'FL from lactose for proper quantitation, has extended to infant formula analysis. Lack of resolution of 2'FL from lactose is in part due to the relatively large concentrations of this disaccharide in human milk and formula (72 ±2.5 g/l, Institute of Medicine 1991). The large quantities of lactose, in turn, generate the *Mass Overload* effect described by Snyder et al. (1997). Under overload conditions, the rapid rate of diffusion of lactose distorts the baseline of the signal generated by 2'FL which elutes nearby. We improved existing HPAEC-PAD methods (represented by "Method 1" in the present account), to optimize 2'FL quantitation by modifying infant formula dilution, using different HPAEC columns and employing different elution solvents and gradients (Method 2). Parameters were chosen to expand a discrete elution window of the chromatogram such that 2'FL was resolved from lactose and other saccharides commonly found in infant formulae such as galactooligosaccharides (GOS) and fructooligosaccharides (FOS). Method 2 allowed for linear quantitation at less than two ppm (< 2 μg/ml).

MATERIALS AND METHODS

Materials: Dionex™ CarboPac™ PA-100 guard columns (4 x 50 mm), CarboPac™ PA-1Analytical columns (4 x 250 mm), CarboPac™ PA-100 Analytical columns (4 x 250 mm), autosampler vials with split septum cap, disposable gold on PTFE electrodes with gaskets, spacer block, Ag/AgCl reference electrodes, and eluent bottles were purchased from Thermo Scientific™ (Grand Island, NY). Nylon membrane filters (0.45μm), 2V filter paper, and polypropylene 50 ml flat bottom centrifuge tubes were purchased from Thermo Scientific™ (Pittsburgh, PA). Water used for all experiments was HPLC grade (Resistivity > 16 MΩ.cm). Low carbonate sodium hydroxide (50% w/w), low carbonate sodium acetate trihydrate, (ACS grade), and glacial acetic acid, (ACS grade) were also purchased from Thermo Scientific™ (Pittsburgh, PA). High purity 2'-Fucosyllactose (2'FL) was purchased from Carbosynth Limited (Compton, England) and Elicityl Oligotech® (Crolles, France). Raffinose pentahydrate was purchased from Millipore® Sigma™ (St. Louis, MO). Two infant formula samples containing 2'Fl were prepared by Perrigo® Nutritionals Product Development (Georgia, Vermont). Table 1 shows the carbohydrate composition of both formulas.

Table 1. Carbohydrate composition of infant formulas used in the study (gm/l).

Ingredient	Formula with 2'FL	Control Formula
Lactose	69.7	71.0
2'Fl	0.230	0
Total Content of scFOS	2.3	1.0
Kestose	0.828	0.36
Nystose	1.17	0.51

Table 1. Two formulas were prepared in the pilot plant with similar protein, lipid and micronutrient composition. one formula was incurred with 2'Fl (prior to thermal treatment) and the other was a standard formula without 2'Fl. Both formulas contained.

HPAEC- PAD was carried out on a Thermo Scientific™ (Grand Island, NY) Dionex™ ICS 5000+ system consisting of an ICS-5000+ SP Quaternary pump, an AS-AP autosampler, an ICS-5000+ DC Detector Compartment, and an ICS-5000 Electrochemical detector. Galactooligosaccharides (GOS, Vivinal® Syrup) were from Friesland Campina Domo® (Amersfoort, The Netherlands) and consisted of saccharides with the general form $(Gal)_nGlc$ in which 33% were of n=1 (lactose), 39% were of n=2 (the trisaccharide O-β-D-galactopyranosyl(1-4)O-β-D-galactopyranosyl(1-4)β-D-glucose), 18% were n=3 (tetrasaccharides with the structure O-β-D-galactopyranosyl-O-β-D-galactopyranosyl(1-4)O-β-D-galactopyranosyl(1-4)β-D-glucose) and the rest were larger galactooligosaccharides. Short chain (scFOS, Nutraflora® L95-S) were from Ingredion™ (Westchester, IL). The main components of scFOS are the trisaccharide *kestose* (β-D-fructofuranosyl(2-1)β-D-fructofuranosyl α-D-glucopyranoside) and the tetrasaccharide *nystose* (β-D-fructofuranosyl(2-1)β-D-fructofuranosyl(2-1)β-D-fructofuranosyl α-D-glucopyranoside). Lacto-N-neotetraose (LNnT) and 3Fucosyllactose (3FL) were from Carbosynth Limited (Compton, England).

Methods: Two elution protocols (termed Method 1 and Method 2) were used to compare the resolution of 2'FL from lactose under different chromatographic conditions. Stock standard solutions for Method 1, containing 400 μg/ml of either 2'FL or raffinose (internal standard), were prepared by dissolving the carbohydrates in water under sonication. Working standards with varying concentrations of 2'FL (4, 8, 20,

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and 40 μg/ml), 16 μg/ml of raffinose (internal standard) and 72 g/l of lactose were prepared under sonication in 50 ml volumetric flasks. Infant formula samples were prepared by adding 2.0 g of powder to 25 ml of water in a 50 ml tube followed by vortex agitation for 20 seconds, and incubation at 50°C for 15 minutes. Fat and denatured protein were removed as described by Vasbinder *et al*., (2003) with modifications. Briefly, 4.0 ml of 0.5 M sodium acetate in 0.5% (v/v) acetic acid was added to the tube containing the formula and mixed. After 10 minutes, the solution was filtered using 2V filter paper. A 3.0 ml aliquot of filtrate and 2.0 ml of stock internal standard solution were added to a 50 ml volumetric flask, diluted with water, and filtered through a 0.22 μm polyethersulfone syringe filter into a vial for HPAEC-PAD analysis. Aliquots of samples or standards (10 μl) were injected onto a Dionex™ CarboPac PA-100 Guard column (4 x 50 mm) connected in series with a Dionex™ CarboPac PA-100 Analytical column (4 x 250 mm) held at 30°C. Chromatographic separation was achieved using three eluents (A: 100 mM NaOH, B: 300 mM Sodium Acetate in 100 mM NaOH, and C: Water) as described in Table 2. Elution was conducted at a flow rate of 1.0 ml/min. Detection (PAD) was performed using a detector compartment temperature of 35°C equipped with gasket, a disposable gold electrode, spacer block, Ag/AgCl reference electrode, and standard quadrupole waveform for carbohydrates.

Table 2. HPAEC-PAD Elution Protocol for Method 1.

Step	Comment	Time (min)	% A	%B	%C
1		0	55	0	45
2	Start gra66dient to 35% B	25	55	0	45
3		35	20	35	45
4	Start gradient to initial conditions	35.1	55	0	45
5		45	55	0	45

Table 2. The elution protocol for Method 1 required three solvents: starting solvent (A): 100 mM NaOH, B: 300 mM sodium acetate in 100 mM NaOH, and C: Water. The columns were equilibrated with starting solvent at the beginning of every run. Flow rate was 1.0 ml/min.

Stock standard solutions for Method 2 consisted of a solution of 440 μg/ml 2'FL in water and raffinose pentahydrate in water (800 μg/ml). Working standards with varying concentrations of 2'FL (1.75, 3.5, 6.6, and 11 μg/ml) and 16 μg/ml of raffinose were prepared. Samples for Method 2 were prepared by adding 1.4 g of infant formula to a 50 ml flat bottom polypropylene centrifuge tube containing 25.0 ml of water. Dissolution and extraction were expedited by vortex mixing for 20 seconds followed by incubation at 50°C for 15 minutes. Fat and denatured protein were removed as described for Method 1 sample preparation above. For Method 2, aliquots of prepared samples or standards (10 μl) were injected onto a Dionex™ CarboPac PA-100 Guard, 4 x 50 mm column in series with a Dionex™ CarboPac PA-1 analytical, 4 x 250 mm column held at 25°C. The separation was performed using three eluents (A: 75 mM NaOH, B: 300 mM Sodium Acetate in 75 mM NaOH, and C: Water) as indicated in Table 3. Elution flow rate was 1.6 ml/min. Detection was performed by PAD as described above for Method 1. To determine elution positions of oligosaccharides that are found in commercially available infant formulas, the following solutions were prepared in water and either injected directly into the chromatograph or used to spike sample solutions: 378 μg/ml of GOS syrup, 35 μg/ml of 3-Fucosyllactose, 103 μg/ml of short chain FOS and 34 μg/ml of the HMO Lacto-N-neotetraose.

Table 3. HPAEC-PAD Elution Protocol for Method 2.

Step	Comment	Time (min)	% A	%B	%C
1		0	8	0	92
2	Start linear gradient to 68% A	2	8	0	92
3		10	68	0	32
4	Start linear gradient to 100% B	32	68	0	32
5		38	0	100	0
6	Start gradient to initial conditions	38.1	8	0	92
7		51	8	0	92

Table 3. The elution protocol for Method 2 required three solvents: starting solvent (A): 75 mM NaOH, (B): 300 mM sodium acetate in 75 mM NaOH, and (C): Water. Flow rate was 1.6 ml/min.

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RESULTS AND DISCUSSION

Figure 1 shows a comparison of two chromatograms resulting from two HPAEC elution protocols (Method 1 and Method 2) for infant formula containing 230 mg/l of 2'FL. The chromatogram from Method 2 (Panel B) shows improved resolution of 2'FL from lactose and demonstrates that scFOS do not interfere with the 2'FL signal. In contrast, Panel A shows incomplete baseline resolution between the lactose peak at retention time 11.9 minutes and 2'FL at retention time 14.8 minutes which is characteristic of HPAEC protocols described by several groups (Erney *et al*., 2000; Thurl *et al*., 2010; Austin *et al*., 2018). A diagonal tracing is required to integrate the 2'FL peak in Method 1 (Fig. ,1 Panel A), while integration in Method 2 (Fig. ,1 Panel B) is easier and cleaner.

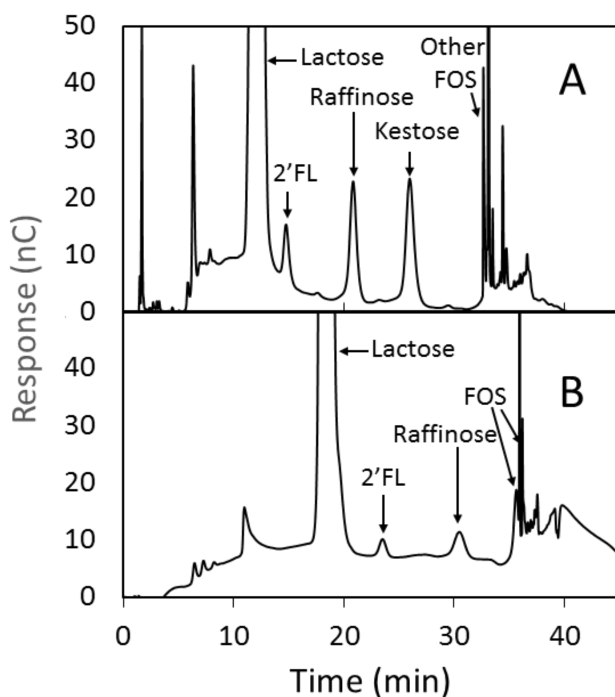

Figure 1. Chromatographic tracings comparing two different HPAEC-PAD protocols. Infant formula containing 2'FL, scFOS and the internal standard raffinose (see Table 1) was reconstituted (2 g in 25 ml), and aliquots were analyzed using Method 1(Panel A). The same formula was reconstituted (1.4 g in 25 ml) and analyzed using Method 2 (Panel B). Carbohydrates were labeled whenever their signals were identified and verified by additional experiments.

This improvement was attained by reducing the concentration of infant formula sample from 2 g/25 ml of diluent to 1.4 g/25 ml (a reduction of 30%), changing the eluent's pH, adding two isocratic holds and replacing the CarboPac PA-100 analytical column of Method 1 by a CarboPac PA-1 analytical column.

Figure 2 depicts chromatograms for a formula devoid of 2'FL (Panel A) and the formula containing 230 mg/l of the oligosaccharide (Panel B). This formula was spiked with GOS and scFOS to demonstrate that 2'FL does not coelute with oligosaccharides commonly found in infant formula (Fig 2, Panel C). In separate experiments we demonstated that the HMO LNnT does not interfere with the 2'FL signal either. Accuracy was characterized by spike and recovery experiments; 2'-FL was spiked (from stock standard) in duplicate at 80 mg/l and 160 mg/l (40 and 80% of intrinsic 2'FL formula content) into a regular (lactose content approximately 70 g/l) infant formula and a low lactose version of the same formula. The spiked formula samples were analyzed in duplicate and recovery was determined by subtracting total measured 2'FL from the intrinsic content of the formula (230 mg/l). Precision was characterized primarily in terms of repeatability and intermediate precision. Three replicates of infant formula were analyzed on three different days. Resulting data from analyses using Method 2 showed the method is precise with intra-day (repeatability) percent relative standard deviations (%RSD) ranging from 0.19% to 1.40% and inter-day (intermediate precision) percent relative standard deviations of 2.03% and 2.11%. The limit of detection (LOD) was estimated based on the signal-to-noise ratio (S/N) of the 2'FL peak obtained from the working standard for multiple injection sequences, the 2'FL concentration corresponding to S/N = 3:1. The average LOD for 2'FL was estimated to be 0.43 µg/ml. The lowest concentration of the standard curve was used for the limit of quantitation (LOQ).

Method 2 is designed to use a four-point calibration curve ranging from 1.75 µg/ml to 11 µg/ml of 2'FL for each analysis. Least squares linear regression was used to calculate the best fit line along with its correlation coefficient. The average correlation coefficient was 0.99951 for all injection sequences. By comparing both panels of Fig. 1, it becomes clear that a specific elution region has been elongated when using Method 2 as opposed to Method 1. It is also noticeable that resolution has been lost after the elution of raffinose since Kestose, a scFOS, is no longer identifiable in Panel B because it elutes at the end of the run with other FOS. Because the

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gradient and analytical column selectively improve resolution near the lactose signal, a shoulder is observed on the tail of the lactose peak indicating the incipient resolution of another carbohydrate. We know that this signal is not due to a contaminant of 2'FL, because the shoulder was also observed in a high lactose infant formula that does not contain 2'FL. The detection of this saccharide that seems to hide under the lactose signal in Method 1 indicates that it is possible to find unidentified components of mixtures by selectively improving resolution in discrete regions of chromatograms. It should be mentioned that one isomer of 2'FL, 3-Fucosyllactose elutes at about 10.5 minutes, prior to lactose elution (data not shown), and no peaks from GOS co-elute with 2'FL or the internal standard raffinose.

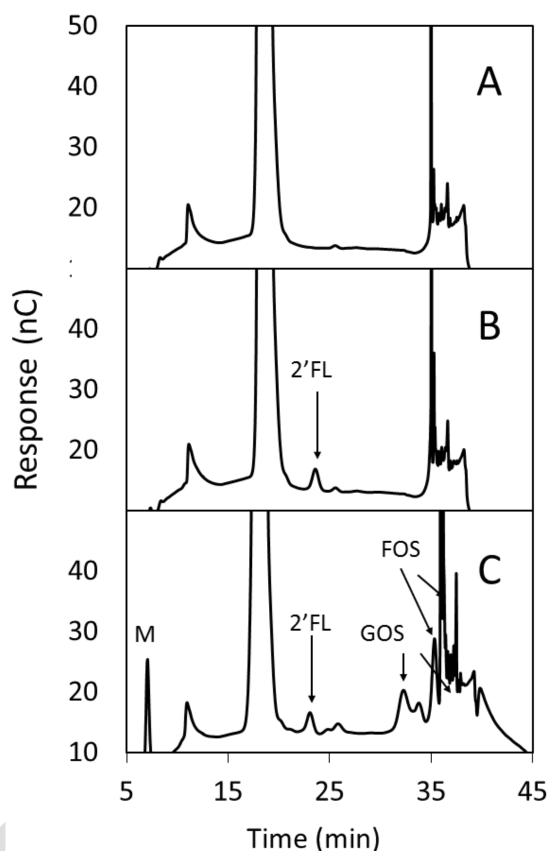

Figure 2. Chromatography of infant formulas using Method 2. Peak elution times for some Panel A, regular infant formula without 2'FL; Panel B, the same formula spiked with 230 mg/l of 2'FL; Panel C, the same formula of Panel B spiked with scFOS and GOS. Elution positions of carbohydrates are indicated by their respective abbreviations except monosaccharides which are denoted by the letter M.

CONCLUSION

Method 2 improves the resolution of 2'FL from the lactose peak in an infant formula matrix. The method was accurate with recoveries ranging from 93.9% to 101.8%. Austin *et al.*, (2018) mention that when a formula containing 2'FL and LNnT prepared in the pilot plant was analyzed using HPAEC-PAD, the obtained data were below expected results which caused them to move to a different chromatographic and detection systems. We also observed similar deficits or quantitation inconsistencies with HPAEC-PAD when using elution protocols that do not result in a clean, baseline separation of 2'FL (Method 1). Method 2, on the other hand, yields expected recoveries presumably because 2'FL is sufficiently resolved from lactose thus limiting its influence on peak shape and distortion of its baseline. Since HPAEC is of common use for the determination of carbohydrates in infant formula, the method does not require the purchase of additional equipment in industrial settings.

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