Customer Application Note 119

Determination of Human Milk Oligosaccharides in Human Breast Milk by HPAE-PAD with On-Line Sample Cleanup

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Key Words
Human Milk Oligosaccharides, Human Breast Milk, HPAE-PAD, On-line Cleanup, Dionex IonPac NG1 Column, Dionex CarboPac PA1 Column

Abstract
In this study, a method for quantification of six human milk oligosaccharides (HMOs), namely 2'-fucosyllactose (2'-FL), 3-fucosyllactose (3-FL), 3'-sialyllactose (3'-SL), 6'-sialyllactose (6'-SL), lacto-N-tetraose (LNT), and lacto-N-neotetraose (LNnT) in breast milk has been developed and validated. The instrument setup was based on the high performance anion-exchange chromatography coupled with pulsed amperometric detection (HPAE-PAD) technique for carbohydrate determinations without sample derivatization. With an on-line sample cleanup using a polymeric reversed-phase column, a "dilute-and-shoot" sample preparation scheme was applied, which significantly reduces sample handling time and offers considerable labor savings and an increase in sample throughput. The results produced by the method were accurate and precise. The method detection limits (MDLs) and the quantification limits (MQLs) for the six HMOs ranged from 4 to 21 µg/mL in milk and 13 to 68 µg/mL in milk, respectively.

Introduction
HMOs are a group of structurally diverse and variable soluble carbohydrates. They constitute the third-largest component of human milk, following lactose and lipids.[1] Several beneficial effects have been associated with HMO, such as immunomodulatory properties and anti-infective effects.[1–5]

By using modern analytical methods, over 130 different types of HMOs have been identified. The concentrations of total and individual oligosaccharides in human milk were found to vary significantly in individuals, depending on factors such as genotype (Lewis and/or Secretor status), ethnicity of the mother,[6] and whether the labor was pre-term or full-term.[7] Production of HMOs is also a dynamic process during the course of lactation where HMOs are present in larger amounts in the first week but concentrations diminish during the proceeding weeks and months.[8]

The most widely used method for quantification of known oligosaccharides is high-performance anion-exchange chromatography coupled with pulsed amperometric detection (HPAE-PAD).[6,9] Due to the presence of interferences in human milk, sample pre-treatment is typically needed before instrumental analysis. General sample preparation includes removing unwanted portions such as lipids and proteins by centrifugation, filtration, or gel permeation chromatography.

In this study, we establish and validate a method for the determination of six key HMOs [2'-fucosyllactose (2'-FL), 3-fucosyllactose (3-FL), 3'-sialyllactose (3'-SL), 6'-sialyllactose (6'-SL), lacto-N-tetraose (LNT), and lacto-N-neotetraose (LNnT)] in human milk with acceptable quantification limits, high throughput, and reduced manual labor. The method can accommodate a wide range of samples in human milk studies where significant variations in concentrations are expected. The method is also applicable for the analysis of conventional milk products for determination of HMO concentrations.

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Materials and Methods

Chemicals

3-FL, 3'-SL, and LNT were from Dextra, UK; 2'-FL and 6'-SL were from Inalco Group (Italy); LNnT was procured from Boehringer Mannheim (Germany). The deionized (DI) water (18.2 MΩ-cm) was produced by a Milli-Q® system from Millipore (USA). Sodium hydroxide solution, 50% w/w, and sodium acetate anhydrous >99% were purchased from Sigma-Aldrich® (USA). Thermo Scientific™ Titan3™ nylon syringe filters were used.

Instrument Setup

A Thermo Scientific Dionex ICS-5000 HPAE-PAD system with dual pumps was used to develop the method. The system configuration is shown in Figure 1.

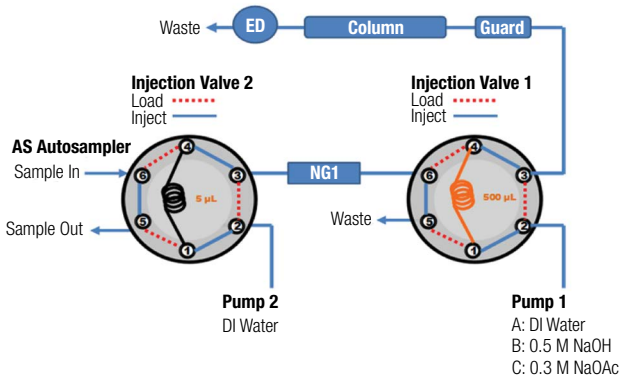

Figure 1. System configuration.

Analytical, guard, and trap columns were Thermo Scientific™ Dionex™ CarboPac™ PA1 column 4 × 250 mm (P/N 035391), Dionex CarboPac PA1 guard column 4 × 50 mm (P/N 043096), and Thermo Scientific™ Dionex™ IonPac™ NG1 column 4 × 35 mm (P/N 039567), respectively.

The instrument parameters were as follows: column temperature: 20°C; injection volume: 5 µL; fraction loop: 500 µL; detection: PAD (Au) Carbohydrate Triple Potential; loading time: 0.8 min. The detailed elution gradient is shown in Table 1.

Sample and Standard Solution Preparation

With the on-line sample cleanup, the sample preparation was basically dilute-and-shoot. For sample analysis, 500 µL of milk sample was pipetted into a 25 mL volumetric flask and diluted to the volume with DI water. For the sample spike study, HMO standards were spiked into a 25 mL volumetric flask with 500 µL of milk sample and then diluted to the volume with DI water. The solution was then filtered through a nylon syringe filter before injection.

A mixed stock solution of six HMOs was prepared at 2000 µg/mL in DI water by dissolving 10 mg of each solid standard in a 5 mL volumetric flask. The working standards were then prepared at eight concentrations (1, 2, 5, 10, 25, 50, 75, and 100 µg/mL) by diluting the mixed stock solution in 25 mL volumetric flasks with DI water.

Results and Discussion

Evaluation of the Dionex IonPac NG1 Column as a Trap

The Dionex IonPac NG1 column was used as a trap column in this study for on-line removal of hydrophobic contaminants from the sample. Ten consecutive injections of a breast milk sample were performed to evaluate the durability and longevity of the trap column. Table 2 shows that multiple injections produced reproducible results. Even over 300 injections during the course of the method development, there was no significant rise in baseline or loss of signal.

Table 1. Elution gradient and valve program.

Time (min)	Pump 1 (1.0 mL/min)				Pump 2 (0.5 mL/min)	Remark
	% A (Deionized Water)	% B (0.5M NaOH)	% C (0.3M NaOAc)	Valve 1 Position	Valve 2 Position (Deionized Water)	
-1.2	80	20	0	Load	Load	Loading of sample to 5 µL sample loop, followed by flushing of the sample from sample loop to NG1 column. These actions are performed by inserting WPS autosampler inject command at -1.2 min.
0	80	20	0	Inject	Inject	Start sample analysis
10	80	20	0	Load	Inject	To bypass 500 µL sample loop and shorten delay volume
18	70	20	10			
28	70	20	10			
32	48	20	32			
39	48	20	32			
39.01	0	20	80			
43	0	20	80			
43.01	80	20	0			
50	80	20	0			

Table 2. Multiple injections of a human milk sample.

Injection Number	Concentration (µg/mL)					
	3-FL	2'-FL	LNnT	LNT	6'-SL	3'-SL
1	29.44	27.17	2.05	19.90	11.28	2.71
2	29.53	27.13	1.97	19.92	11.27	2.69
3	29.36	26.99	2.00	19.83	11.22	2.65
4	29.30	27.08	2.01	19.87	11.18	2.69
5	29.34	27.01	2.01	19.83	11.18	2.68
6	29.25	26.91	1.99	19.86	11.13	2.73
7	29.23	26.87	2.00	19.55	11.15	2.69
8	29.27	26.93	2.00	19.71	11.12	2.68
9	29.20	26.93	1.99	19.62	11.10	2.63
10	29.20	26.79	1.98	19.50	11.09	2.71
Mean	**29.31**	**26.98**	**2.00**	**19.76**	**11.17**	**2.69**
SD	**0.11**	**0.12**	**0.02**	**0.15**	**0.07**	**0.03**
RSD	**0.37**	**0.45**	**1.02**	**0.77**	**0.59**	**1.08**

In the event that the performance of the Dionex IonPac NG1 column deteriorates due to contamination (e.g. an elevated baseline), it can be regenerated using a freshly prepared cleanup solution of 200 mM HCl in 80% acetonitrile flowing for 1 h at 0.5 mL/min. Regeneration is performed when the column is out of the analytical flow path.

Linearity

An 8-level external calibration for each of the target analytes (1, 2, 5, 10, 25, 50, 75, and 100 µg/mL in DI water) was made to cover the wide range of variations that would be expected for a human milk study with a large sample size. The chromatograms of the standards are displayed in an overlaid pattern in Figure 2. It shows a flat baseline, good peak shapes, and no retention time shift, thereby demonstrating good method and system reproducibility.

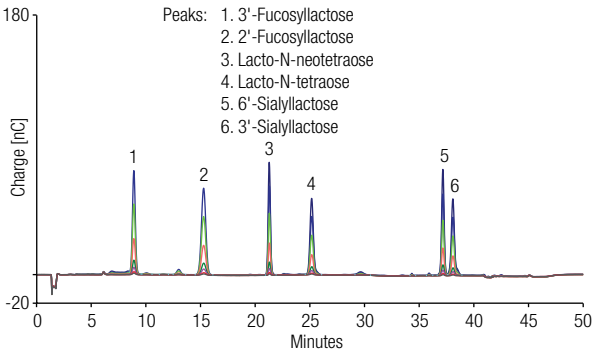

Figure 2. Overlay of chromatograms of standards.

The calibration curve was plotted with 1/× weighting on 16 points (bracket calibration). All components show >99.5% coefficient of determination (multiplied by 100%) in the concentration range of 1 to 100 ppm (Table 3). Figure 3 shows that from 1 µg/mL to 100 µg/mL, the calibration residual errors for all compounds were below 10% except for 3'-SL, which was 15% at 1 µg/mL. This higher residual error may be caused by the lower response of this compound compared to most other compounds. With a lower response, the peak for 1 µg/mL 3'-SL will be closer to the baseline, which naturally fluctuates at this low level of response. Most calibration points above 10 µg/mL have a residual error less than 5%.

Table 3. Calibration of HMOs of Day 1.

HMO	Ret. Time (min)	Cal. Type	Points	Coeff. Det. (%)	Offset	Slope
3-FL	8.9	XLOff	16	99.9161	0.0355	0.2853
2'-FL	15.3	XLOff	16	99.9275	0.006	0.3771
LNnT	21.3	XLOff	16	99.8497	0.0393	0.1964
LNT	25.2	XLOff	16	99.8894	-0.0019	0.1937
6'-SL	37.2	XLOff	16	99.9014	-0.0186	0.1934
3'-SL	38.1	XLOff	16	99.9025	-0.0364	0.1781

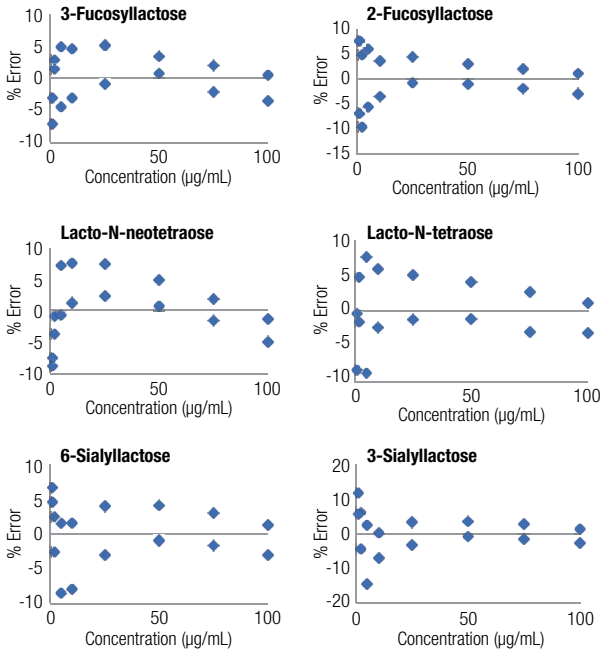

Figure 3. Calibration residual errors.

Method Detection Limit and Method Quantification Limit

MDL and MQL were established by analyzing eight spiked matrix blanks. As practically there is no HMO-free human milk available, two whole milk powder materials were used as the matrix blanks, although there were small signals around the elution time of 3-FL and 3'-SL (Figure 4).

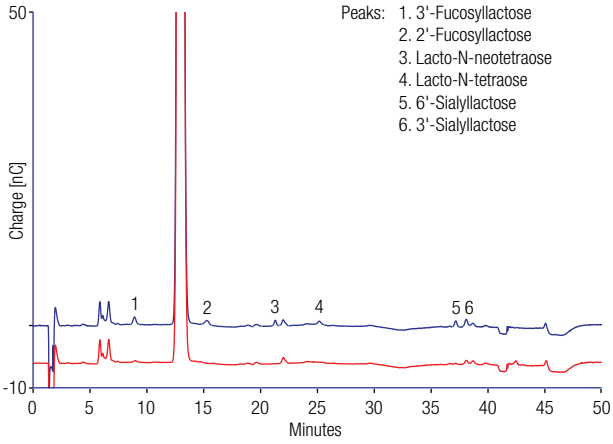

Figure 4. Whole milk powder spike (upper trace: sample spike; lower trace: sample).

Milk powder was reconstituted to liquid form (2.5 g of powder in 25 mL volumetric flask with DI water), and the milk solution was further diluted 50 fold (the same dilution factor as that for human milk samples), and spiked with standards at approximately 1 µg/mL. The solution was then filtered through a nylon syringe filter before injection. Four independent replicates of the unspiked and the spiked samples for each matrix were run to determine the standard deviation (SD) of the recovered amount. The MDL was calculated by multiplying SD by a factor of 3, and MQL was 3 times the MDL. The results and calculations are detailed in Table 4. HMOs are normally present in breast milk of secretors at a mean level of tens to thousands µg/mL milk.[6] Thus, MDL and MQL values established here are more than adequate for human milk analysis.

Table 4. MDL and MQL calculations.

Matrix Blank Spike	Concentration (µg/mL)					
	3-FL	2'-FL	LNnT	LNT	6'-SL	3'-SL
Spike 1_1	1.03	0.75	0.80	0.91	1.41	0.76
Spike 1_2	1.08	0.80	0.83	0.91	1.41	0.87
Spike 1_3	1.05	0.81	0.85	0.77	1.41	1.04
Spike 1_4	1.05	0.74	0.84	0.87	1.38	1.02
Spike 2_1	0.98	0.99	0.88	0.88	1.10	0.74
Spike 2_2	1.00	0.92	0.83	0.74	1.16	0.82
Spike 2_3	1.04	0.92	0.82	0.98	1.19	1.02
Spike 2_4	1.04	0.89	0.85	0.91	1.16	0.99
Mean	**1.03**	**0.85**	**0.84**	**0.87**	**1.28**	**0.91**
SD	**0.03**	**0.09**	**0.03**	**0.08**	**0.14**	**0.13**
MDL (µg/mL solution)	**0.09**	**0.27**	**0.08**	**0.24**	**0.41**	**0.38**
MDL (µg/mL milk)	**4.5**	**15**	**4**	**12**	**21**	**19**
MQL (µg/mL solution)	**0.32**	**0.9**	**0.26**	**0.79**	**1.4**	**1.4**
MQL (µg/mL milk)	**16**	**45**	**13**	**40**	**68**	**63**

Accuracy and Precision

Accuracy and precision were determined by analyzing recoveries of six spiked pooled breast milk samples (three colostrum and three mature milk samples). Each pooled sample contained milk from various donors (one to thirty donors in one sample). Before spiking, milk samples were analyzed to determine the native concentrations of HMOs, and the samples were then spiked with HMO standards at the concentrations approximate to those pre-existing levels in the samples. The experiments were conducted in three days. The chromatograms of sample and sample spike are displayed in Figures 5a and 5b. The spiking concentrations are displayed in Table 5.

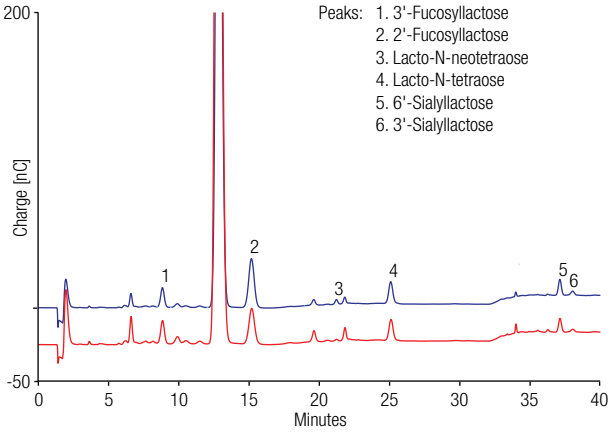

Figure 5a. Chromatograms of sample and sample spike for breast milk (upper trace: sample spike; lower trace: sample).

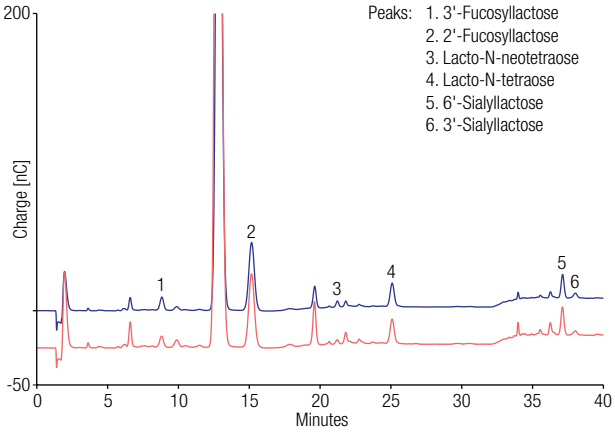

Figure 5b. Chromatograms of sample and sample spike for colostrum (upper trace: sample spike; lower trace: sample).

Table 5. Spiking concentrations.

	3-FL	2'-FL	LNnT	LNT	6'-SL	3'-SL
Concentration (µg/mL in analyzed solution)	10	50	5	25	15	5
Concentration (mg/L in milk)	500	2500	250	1250	750	250

The recoveries of all components were within 89–112% (Table 6). The RSDs for the six compounds were all < 6%.

Table 6. Spike recoveries.

Sample Reference Number*	Recovery (%)					
	3-FL	2'-FL	LNnT	LNT	6'-SL	3'-SL
BM P61379 (Day 1)	99	98	110	108	101	95
BM R31207 (Day 2)	102	93	111	110	104	91
BM P48299 (Day 2)	102	97	112	108	105	89
CL T4139 (Day 1)	106	95	110	111	105	101
CL R31208 (Day 3)	102	93	111	107	101	101
CL R31209 (Day 3)	103	96	109	106	102	99
Mean	**102**	**95**	**111**	**108**	**103**	**96**
SD	**2.25**	**2.07**	**1.05**	**1.86**	**1.90**	**5.18**
%RSD	**2.20**	**2.17**	**0.95**	**1.72**	**1.84**	**5.39**

* BM = mature breast milk >4 weeks after giving birth; CL = colostrum <1 week after giving birth.

Conclusion

This study demonstrates that a method for accurate determination of HMOs in human breast milk has been successfully established. With on-line sample cleanup by the Dionex IonPac NG1 column, sample handling time is significantly reduced, offering considerable savings in labor and an increase in throughput. The results produced by the method are reliable with a high degree of accuracy and precision. MDLs and MQLs for the six HMOs ranged from 4 to 21 µg/mL in milk and 13 to 68 µg/mL in milk, respectively, and meet the requirements for a human milk study.

Acknowledgement

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Thermo Scientific Comments

If you choose to set up this application, we recommend using the 4-potential carbohydrate waveform (Waveform A in Thermo Scientific Dionex Technical Note 21). We also do not recommend having an eluent bottle with only sodium acetate as that can lead to microbial growth and system contamination (Thermo Scientific Dionex Technical Note 71). We recommend making eluent C 100 mM NaOH/sodium acetate) and in every step that has eluent C eliminate the 20% eluent B and add 20% to eluent A (e.g. a 70:20:10 step would become 90:0:10).

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