

## Benefits of MaxPeak™ High Performance Surfaces in the Determination of $\beta$ -Galactooligosaccharides in Infant Formula by HILIC

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### Abstract

Oligosaccharides, such as  $\beta$ -galactooligosaccharides (GOS), are often formulated in foods and beverages as prebiotics. We participated in a multi-lab testing (MLT) for the evaluation of an international standard (AOAC Official Method 2021.01) on the determination of GOS in infant formula by hydrophilic interaction liquid chromatography (HILIC). Excellent results were obtained using an ACQUITY™ UPLC™ H-Class System coupled with an ACQUITY UPLC Fluorescence Detector and an ACQUITY UPLC Glycan BEH™ Amide Column. We also uncovered issues of analyte loss and carry-over in the HILIC of dextran oligosaccharides (which was used in the method for molecular weight determination) and demonstrated that these issues were effectively resolved by using the MaxPeak High Performance Surfaces (HPS) incorporated system and column, *i.e.*, an Arc™ Premier System and an XBridge™ Premier Glycan BEH Amide Column. Considering the structural similarity between dextran and GOS, it is prudent to use MaxPeak HPS incorporated systems and columns in the HILIC analysis of GOS to eliminate potential issues.

## Benefits

MaxPeak HPS technology helps to resolve issues of analyte loss and carry-over in HILIC of dextran oligosaccharides ( $DP \geq 6$ ).

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## Introduction

The  $\beta$ -galactooligosaccharides (GOS) are oligosaccharides that each comprises a chain of galactose units with an optional terminal glucose unit. They occur naturally in human milk and the milk of many animals. GOS are resistant to hydrolysis by human digestion but can be consumed by bifidobacteria and lactobacilli in the colon. Therefore, they are prebiotics and provide health benefits to human. Many food products are formulated or supplemented with GOS to enhance their health values to consumers.

The content of GOS in foods can be determined by different techniques, such as high-performance anion exchange chromatography (HPEAC),<sup>1,2</sup> capillary electrophoresis (CE),<sup>3</sup> and hydrophilic interaction liquid chromatography (HILIC).<sup>4,5</sup> Recently, a HILIC method has been approved as an AOAC Official Method (AOAC 2021.01 Final Action)<sup>6</sup> for infant formula. We participated in a multi-lab testing (MLT) for the evaluation and approval of this standard method.

In this application note, we demonstrate the analytical performance of the AOAC Official Method (AOAC 2021.01) for the determination of GOS in infant formula using an ACQUITY UPLC H-Class PLUS System coupled with an ACQUITY UPLC FLR Detector and an ACQUITY UPLC Glycan BEH Amide Column. We also present findings in the HILIC of dextran oligosaccharides (Dextran Calibration Ladder), which was used in the GOS analysis for molecular weight and describe the potential benefits of MaxPeak HPS for the analysis of oligosaccharides by HILIC.

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## Experimental

The chemicals and sample preparation procedures that are recommended in AOAC 2021.01 are used in this study.

### Standards and Reagents

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Maltotriose ( $\geq 90\%$  HPLC), 2-methylpyridine borane complex (2-picoline borane, 95%), amyloglucosidase (from *Aspergillus niger*), Dextran (from *Leuconostoc mesenteroides*, Mw 1,000), glacial acetic acid (anhydrous), sodium hydroxide pellets, Dimethylsulfoxide (puriss. p.a.), acetonitrile (LC-MS grade), formic acid (reagent), ammonium hydroxide solutions (ACS reagent) were purchased from Sigma-Aldrich (St Louis, MO, USA), laminaritriose ( $>90\%$ ) and  $\beta$ -galactosidase (from *Aspergillus niger*, 4000 U/mL) were from Megazyme (Bray, Ireland), 2-aminobenzamide (2-AB, 98%) was from TCI (Tokyo Chemical Industry Co., Japan). Deionized water ( $18 \text{ M}\Omega\cdot\text{cm}$ ) was prepared in-house. Infant formula and adult nutritional samples in powder and ready-to-feed liquid forms were provided by the AOAC 2021.01 MLT organizer.

### Sample Preparation

Infant formula samples were prepared first as ready-to-feed liquid, *i.e.*, weigh 25 g of infant formula powder into a bottle and add water to a final total weight of 225 g. Place the mixture in a water bath at  $70^\circ\text{C}$  for 25 min under constant stirring. Cool the solution to room temperature. Then, weigh about 4.5 g of this sample into a 25-mL volumetric flask and dilute with water to the mark. Take two aliquots of each diluted sample ( $500 \mu\text{L}$ ) into two microtubes (1.5 mL) marked *A1* (assay 1) and *A2* (assay 2), add  $200 \mu\text{L}$  of amyloglucosidase solution ( $60 \text{ U/mL}$  in  $0.2 \text{ M}$  sodium acetate buffer pH 4.5) in both tubes. Add  $50 \mu\text{L}$  of water in tube marked *A1* and  $50 \mu\text{L}$  of  $\beta$ -galactosidase solution ( $4000 \text{ U/mL}$ ) in tube marked *A2*. Mix (Vortex) and place in a water bath at  $60^\circ\text{C}$  for 2 hours  $\pm 5$  min. At the end of the incubation time, put all *A2* tubes (containing  $\beta$ -galactosidase) in a boiling water bath for 5–6 min to stop the reaction. Then mix (Vortex) and place at  $4^\circ\text{C}$  for 5–10 min. Add  $100 \mu\text{L}$  of internal standard (I.S.) laminaritriose ( $2.0 \mu\text{mol/mL}$ ) in all tubes and mix well.

### Derivatization (2-AB)

Transfer  $20 \mu\text{L}$  of solutions (both *A1* and *A2*) into 2-mL microtubes, add  $100 \mu\text{L}$  of water and  $100 \mu\text{L}$  of 2-AB labeling reagent ( $0.35 \text{ M}$  2-AB,  $1 \text{ M}$  2-picoline borane in 30 vol% acetic acid in DMSO) to each tube. Mix and place the tubes in a water bath at  $65^\circ\text{C} \pm 1^\circ\text{C}$  for 1 h  $\pm 5$  min. After 1 h, the tubes are then placed at  $4^\circ\text{C}$  for 5–10 min. Once cooled, centrifuge for 10–20 seconds at  $10,000 \times g$ , dilute with 1 mL of acetonitrile:water (75/25 v/v) solution. Mix well, then centrifuge for 5 min at  $10,000 \times g$  before transferring 1 mL of supernatant to an injection vial.

### Standard Solutions

Weigh 50 mg of maltotriose (recorded the mass to 0.1 mg) and transfer into a 10-mL volumetric flask, dilute to the volume with water. Prepare calibration solutions at 6 levels (40, 200, 400, 800, 1200, 1600

nmol/mL) by diluting the maltoriose stock solution with water. For each of the calibration standard solution, transfer 500  $\mu$ L into a microtube (1.5 mL). Add 250  $\mu$ L of water. Mix and place in a water bath at 60 °C for two hours. At the end of the incubation time, mix and place at 4 °C for five to ten minutes, add 100  $\mu$ L of I.S., then continue with the standards on derivatization (2-AB) step (previous section).

## LC Conditions

System:	ACQUITY UPLC H-Class PLUS System (or Arc Premier System with BSM) and an ACQUITY UPLC FLR Detector
Software:	Empower™ 3 Chromatography Data Software
Column:	ACQUITY UPLC Glycan BEH Amide Column, 1.7 $\mu$ m 2.1 x 150 mm (186004742), or XBridge Premier Glycan BEH Amide Column, 2.5 $\mu$ m, 2.1 x 150 mm (186009943)
Vial:	PP vial 700 $\mu$ L volume with cap and preslit septum (p/n: 186005221)
Temperature:	25 $\pm$ 2 °C
Injection volume:	2 $\mu$ L
Run time:	60 minutes
Mobile phase A:	Acetonitrile
Mobile phase B:	100 mM ammonium formate pH 4.40 $\pm$ 0.05
FLR detector:	Excitation $\lambda$ = 330 nm; Emission $\lambda$ = 420 nm

## UPLC Gradient Elution Program

Time (min)	Flow (mL/min)	%A	%B
0	0.6	88.0	12.0
7.0	0.6	88.0	12.0
17.0	0.6	85.0	15.0
21.0	0.6	85.0	15.0
36.0	0.6	72.6	27.4
44.0	0.6	54.0	46.0
44.1	0.3	54.0	46.0
44.5	0.3	30.0	70.0
49.5	0.3	30.0	70.0
52.0	0.3	88.0	12.0
54.0	0.6	88.0	12.0
60.0	0.6	88.0	12.0

## Molecular Weight Determination by LC-MS

MS system: ACQUITY QDa™ Mass Detector\*(Performance)

Capillary voltage: 0.8 kV

Ion polarity: Negative

Probe temperature: 600 °C

Sampling rate: 5 points/sec

Gain: 1

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*\*Equivalent and superior performance expected with the Waters ACQUITY QDa II Mass Detector*

## SIR Channel

GOS	Start (min)	Stop (min)	SIR mass (Da)	GOS MW (g/mol)	Cone voltage (V)
Hex <sub>2</sub> -2AB	3.0	15.0	461.3	342	25
Hex <sub>3</sub> -2AB	13.0	28.0	623.4	504	35
Hex <sub>4</sub> -2AB	27.0	33.5	785.4	666	55
Hex <sub>5</sub> -2AB	32.5	38.5	947.4	828	60
Hex <sub>6</sub> -2AB	36.0	44.0	1109.5	990	75

### Calculations

In this method, samples are treated with amyloglucosidase to hydrolyze maltodextrins in the sample ( *assay 1*). Then samples are further treated with  $\beta$ -galactosidase (*assay 2*) to hydrolyze the GOS in samples. The content of GOS ( $C_{GOS}$ , Eq. 3) is calculated as the difference between the oligosaccharide content determined from assay 1 ( $C_{assay1}$ , Eq. 1) and assay 2 ( $C_{assay2}$ , Eq. 2) as shown below:

$$C_{assay1} = \sum (C_m \times MW) \times V/m \times 0.0001 \quad (\text{Eq. 1})$$

$$C_{assay2} = \sum (C_m \times MW) \times V/m \times 0.0001 \quad (\text{Eq. 2})$$

$$C_{GOS} = C_{assay1} - C_{assay2} \quad (\text{Eq. 3})$$

Where  $C_m$  is the molar concentration (in  $\mu\text{mol/mL}$ ) of each oligosaccharide in the chromatogram. MW is the molecular weight of each oligosaccharide; V is the volume to which the original sample weight was diluted (in mL); m is the weight of sample diluted to volume (V) (in g); and 0.0001 is the factor to convert the results from  $\mu\text{g/g}$  to  $\text{g}/100\text{g}$ .

## Molecular Weight Determination Using Dextran

The MW of oligosaccharides can be determined from LC-MS. If a mass spectrometer is not available, AOAC Official Method 2021.01 provides an alternative way to determine MW using a dextran standard (Dextran Calibration Ladder). Dextran Calibration Ladder is composed of a group of dextran oligosaccharides, which elute from smallest to the largest under the method conditions. The first peak is isomaltose, which is composed of 2 glucose units (GU), the next peak of dextran has a GU of 3, and so on. The GU of each of the dextran peaks is then plotted against its relative retention time (RRT; relative to I.S.), and these data points are fitted to a third order polynomial equation, which is then used to calculate the GU values of peaks from their RRTs in the analysis of unknown samples. From the calculated GU value, the number of hexose units (or degree of polymerization, DP) is assigned using a table provided in the AOAC Official Method 2021.01 (Table AOAC 2021.01.E). Then the MW of oligosaccharides are calculated from the DP.

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## Results and Discussion

Excellent analytical performance in linearity, sensitivity, repeatability, and accuracy has been obtained in the determination of GOS in infant formula (AOAC Official Method 2021.01) using an ACQUITY UPLC H-Class System coupled with an ACQUITY UPLC FLR Detector and an ACQUITY UPLC Glycan BEH Amide Column.

### Calibration Linearity

The relationship between the peak area ratio (maltotriose/I.S.) and the maltotriose molar concentration (nmol/mL) is shown in Figure 1. A linear model by the least squares regression fitted the data well with coefficient of determination ( $R^2$ ) of 0.99998. This calibration curve worked for all oligosaccharides because they all had the same response factor (each molecule had one 2-AB label and thus had the same fluorescence signal).

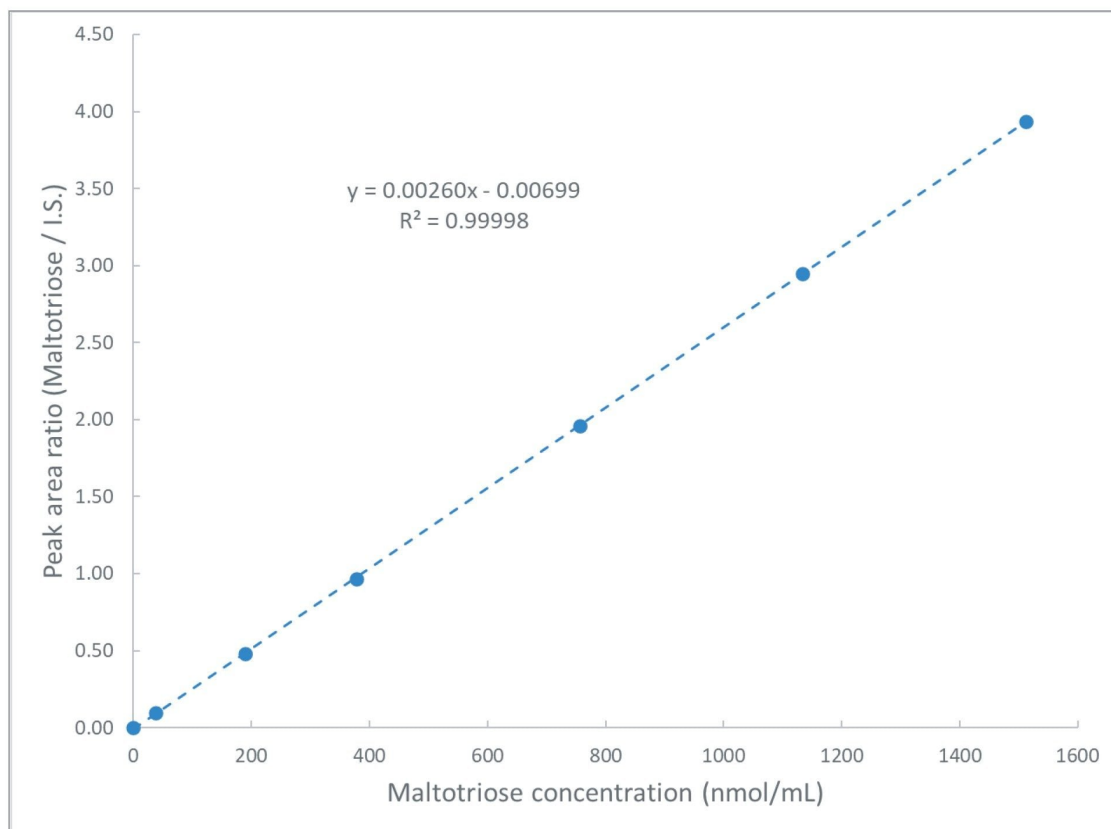


Figure 1. Calibration plot showing a linear relationship between the peak area ratio (maltotriose to I.S.) and molar concentration of maltotriose. Fitted equation and  $R^2$  (0.99998) are shown in the plot.

### Sensitivity

The limit of quantitation (LOQ) was estimated at the 10 times of the standard deviation (SD) of the responses of the lowest concentration standard solution (n=5) assuming a MW of 342 g/mol and 5 g sample amount. The estimated LOQ values was 0.003 g/100 g, which was within the expectation of the method performance.

### Repeatability and Accuracy

The repeatability was assessed using the two infant formula practice samples in the MLT. These samples were measured two times each day on two different days (See Table 1). Relative standard deviations (RSD) of less than 1.0% were obtained for these two samples, demonstrating a good repeatability. The



mean values of these two samples were also compared to the average values provided in the MLT report in Table 1.<sup>7</sup> Differences of 3.4% and -6.4% were obtained, which are within the performance requirement (within  $\pm 10\%$ ).

Sample	Measured GOS content (g/100 g)				Ref. Conc <sup>1</sup> (g/100g)	Diff. <sup>2</sup> (%)
	Day 1	Day 2	Mean	RSD (n=4)		
A	0.272	0.266	0.270	0.9%	0.261	3.4%
	0.271	0.270				
B	0.190	0.187	0.188	1.0%	0.201	-6.4%
	0.185	0.189				

Note: 1) Reference concentration values were the mean values in the multiple lab study.  
2) Difference between the Mean and the reference concentration.

Table 1. Repeatability and accuracy in the determination of GOS in infant formula.

### Sample Analysis

Figure 2. shows typical chromatograms obtained in the determination of GOS in infant formula. The retention time windows for different hexose units (the same as DP) are marked in these chromatograms. GOS content was calculated from the difference between assay 1 and assay 2 results. Table 2. shows a comparison of GOS content determined in our lab and the average values and reproducibility ( $RSD_R$ ) reported in the MLT.<sup>7</sup> The relative differences between our lab's results and the average MLT results (Rel. Diff. in Table 2) were well within the corresponding  $RSD_R$ , with the exception of sample G, which was slightly larger than its  $RSD_R$ . These results demonstrated excellent accuracy in the analysis of GOS content in these MLT samples using the ACQUITY UPLC H-Class PLUS System.

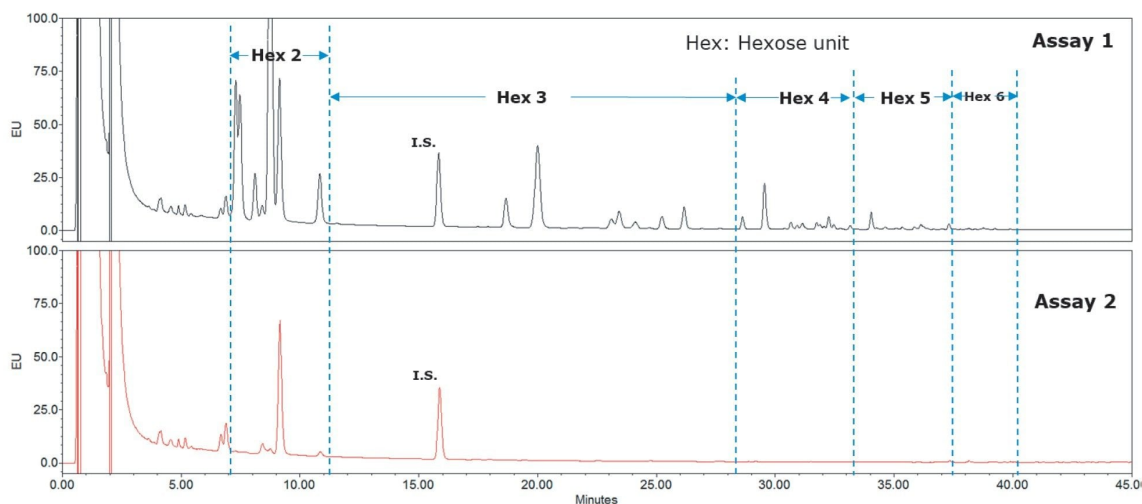


Figure 2. Chromatograms for a typical infant formula sample from Assay1 and Assay 2. The retention time windows for oligosaccharides with hexose unit two to six are indicated in chromatograms.

Samples	Ref. value <sup>1</sup> (g/100 g)	Measured (g/100 g)	Rel. diff.	RSD <sub>R</sub> <sup>2</sup>
C	0.59	0.61	3.4%	10.3%
D	0.69	0.73	5.8%	9.7%
E	0.62	0.66	6.5%	8.1%
F	0.86	0.87	1.2%	9.5%
G	0.32	0.33	3.1%	10.9%
H	0.24	0.27	12.5%	11.6%
I	0.84	0.90	7.1%	12.1%

Note: 1) Average GOS contents obtained in MLT.

2) Reproducibility obtained in MLT.

Table 2. Sample analysis results.

## Comparison Study for Impact of MaxPeak High Performance Surfaces

MaxPeak HPS Technology has been found very useful in mitigating issues such as analyte loss, carry-over, and peak tailing that are related to metal analyte interactions in LC.<sup>8-12</sup> The effects of MaxPeak HPS for the analysis of GOS were investigated using a side-by-side comparison approach. Two LC system and column configurations were configured. One consisted of an ACQUITY H-Class PLUS System and an ACQUITY UPLC Glycan BEH Amide Column (2.5  $\mu\text{m}$  2.1 x 150 mm) and is hereon referred to as a “*Conventional Configuration*” . The other consisted of an Arc Premier System with an XBridge Premier Glycan BEH Amide Column (2.5  $\mu\text{m}$ , 2.1 x 150 mm) and is hereon referred to as an “*HPS Configuration*” . The primary difference between these two Configurations is the incorporation of MaxPeak HPS technology within the system and column in the *HPS Configuration*.

## Issues in HILIC Analysis of Dextran

### Analyte Loss

In this method, 2-AB labeled dextran (Dextran Calibration Ladder) was chromatographed under the same conditions as used in GOS analysis to calibrate the column for MW. Figure 3. shows a comparison of HILIC-FLR chromatograms obtained on the *Conventional Configuration* and the *HPS Configuration* for a 2-AB labeled dextran (with an I.S.). In Fig. 3, one can see that some dextran oligosaccharides (DP 6 and higher) in the *Conventional Configuration* chromatogram are smaller in peak height than the corresponding peaks (with the same DP) in the *HPS Configuration* chromatogram. Additional investigation with repeated injections revealed that inconsistent (from injection to injection) and smaller peak areas were obtained on the *Conventional Configuration*, while consistent and higher peak areas were obtained on the *HPS Configuration* for these dextran oligosaccharides (DP 6 and higher). For a better comparison, the peak areas from the *Conventional Configuration* were normalized to those from the *HPS Configuration*, and the relative peak areas (relative to the *HPS Configuration* peak areas) were plotted in Figure 4. Results from the first, 18<sup>th</sup>, and 21<sup>st</sup> injections were included in Figure 4 to show trends from injection to injection. In Figure 4, one can see that oligosaccharides started to drop from 100% relative peak area (loss analyte in chromatography) at DP 6, and the higher the DP, the more the analyte loss. Also, among the repeated injections, there seemed to be a trend that the extent of the analyte loss was alleviated with repeated injections. The more the injection number, the less the analyte loss on the *Conventional Configuration*. However, the loss was still significant even after 20 injections (30% for the DP 11 peak). On the other hand, there was no analyte loss for small dextran oligosaccharides (DP 3 to 5). These observations indicate that the analyte loss that occurred to dextran oligosaccharides of DP 6 and higher is mainly related to the increased chain length (or MW) of dextran, not the functional groups in the 2-AB label.

## Carry-over

Carry-over peaks, or residue peaks from the previous injection, were found only on the *Conventional Configuration* for dextran oligosaccharides with DP 6 and higher (See Figure 5). And no carry-over was detected on the *HPS Configuration* for any dextran oligosaccharide. The extent of carry-over on the *Conventional Configuration* was also found to increase with the increase in DP of oligosaccharide. In summary, the facts that no carry-over nor analyte loss was found on the *HPS Configuration* for any dextran oligosaccharides demonstrated that MaxPeak HPS technology effectively resolves the analyte loss and carry-over issues for dextran oligosaccharides that were clearly observed on the conventional system and column.

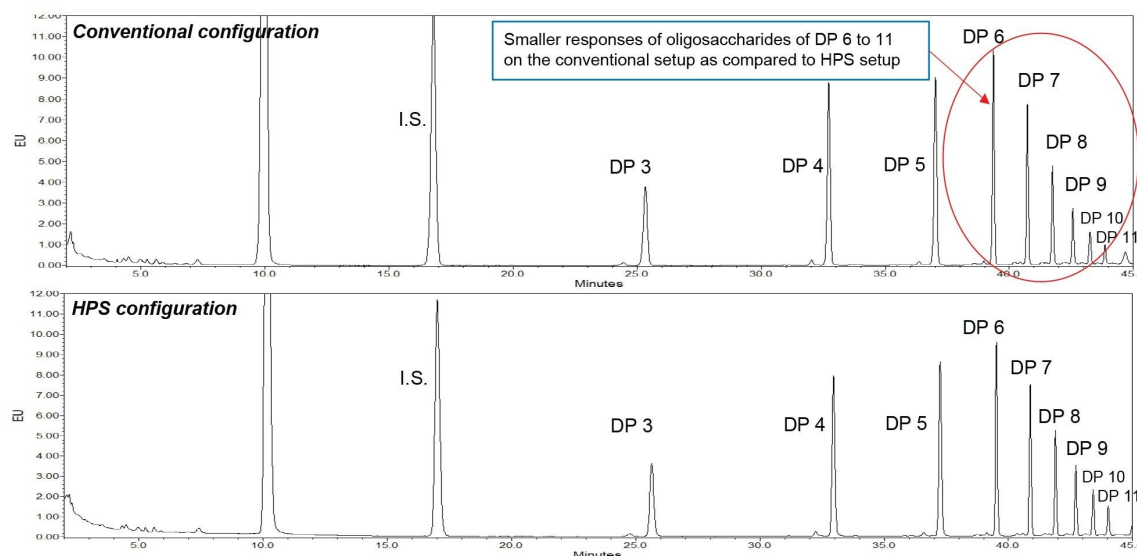


Figure 3. Comparison of HILIC-FLR chromatograms of Dextran Ladder (2-AB labeled) obtained from the Conventional and the HPS Configurations.

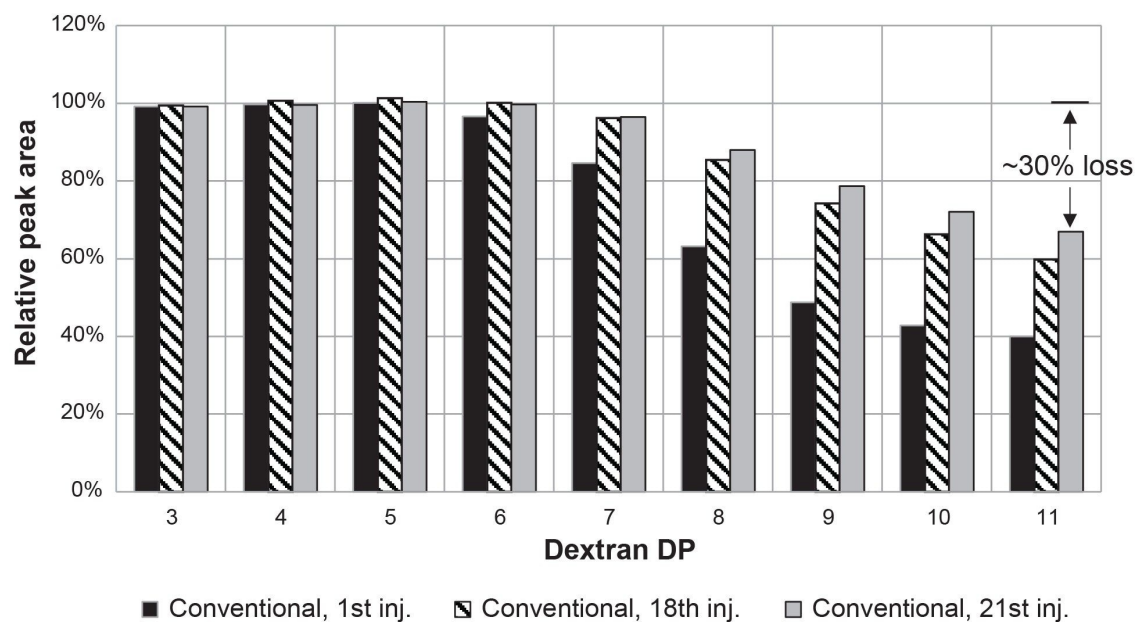


Figure 4. Loss of analyte in dextran oligosaccharides (DP  $\geq$  6) obtained on conventional system and column as compared to those obtained on MaxPeak HPS-incorporated System and Column (set at 100%).

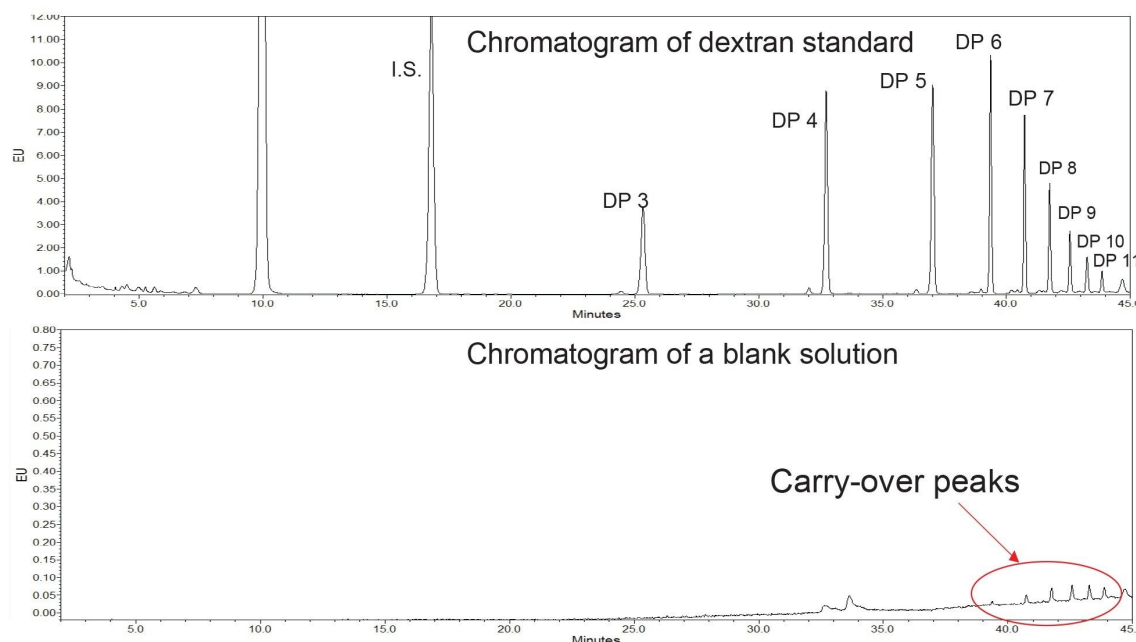


Figure 5. Carry-over peaks observed in HILIC-FLR chromatogram of a blank solution following an injection of a dextran standard on a conventional system and column.

### Effects of MaxPeak HPS in the Determination of GOS in MLT Samples

The MLT samples were analyzed using the *Conventional* and the *HPS Configurations*. Interestingly, no difference was found in GOS results between these two Configurations. Inspection of the chromatograms (assay 1) showed that most of the GOS peaks were in the retention time windows for DP 5 or less. There was little GOS of DP 6 or higher in samples after enzymatic hydrolysis of maltodextrins (see Figure 2. Assay 1). It is not clear whether any analyte loss or carry-over issue could occur to GOS under these HILIC conditions or not. Further investigation with GOS of high DP ( $DP \geq 6$ ) would be needed for confirmation. Nevertheless, considering the similarity between dextran and GOS in their monomers (glucose and galactose), it would be prudent to use a MaxPeak HPS-incorporated system and column in the GOS analysis to avoid potential issues of error due to analyte loss or carry-over.

## Conclusion

We demonstrated the excellent analytical performance in the determination of GOS in infant formula using an ACQUITY H-Class PLUS System coupled with an ACQUITY FLR Detector and an ACQUITY UPLC Glycan BEH Amide Column. We uncovered, for the first time, that dextran oligosaccharides (2-AB labeled) with DP of six or higher suffered analyte loss and carry-over issues under certain HILIC conditions. Using a MaxPeak HPS-incorporated system and column Configuration (Arc Premier System and XBridge Premier Glycan BEH Amide Column), the analyte loss and carry-over issues were effectively resolved. Although no evidence of analyte loss was found in the determination of GOS using the conventional system and column Configuration, considering the similarity in chemical structures between the dextran oligosaccharides and GOS, it would be prudent to employ the MaxPeak HPS incorporated systems and columns for the GOS analysis to avoid any potential issues.

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