

# Profiling galactosyloligosaccharide-containing samples by high-performance anion-exchange chromatography with pulsed amperometric detection (HPAE-PAD)

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## Goal

To develop an HPAE-PAD method for profiling Bimuno GOS powder and Bimuno GOS syrup

## Introduction

Prebiotics are non-digestible ingredients that beneficially affect human health by selectively stimulating the growth of one or a limited number of bacteria in the colon.<sup>1</sup> Food ingredients that meet this definition are water-soluble carbohydrates such as galactosyloligosaccharides (GOS), fructosyloligosaccharides (FOS), and inulin. Certain GOS occur naturally in the milk of many animals including humans,<sup>2</sup> cows, and wallabies.<sup>3</sup> GOS are primarily composed of galactose and often terminate with a glucose residue at the reducing end. These carbohydrates are enzymatically produced by transgalactosylation reactions of lactose catalyzed by  $\beta$ -galactosidases and give rise to galactose (Gal) oligomers with a terminal glucose that features different glycosidic linkages and degrees of polymerization (DP). For example, Vivinal® GOS (a commercial GOS



product) has predominantly (1→4)-linked  $\beta$ -D-Gal in the oligosaccharides, and fewer linkages such as (1→6)-linked  $\beta$ -D-Gal and (1→3)-linked  $\beta$ -D-Gal are observed.<sup>4</sup> Depending on the enzymatic source used for their synthesis, the chemical structure of these oligosaccharides varies<sup>5</sup> and, consequently, their effect on gut microflora can change. Many studies have investigated the effect of dietary GOS on gastrointestinal microflora in infants. The consumption of GOS-supplemented infant formulas is consistently reported to increase the bifidobacteria populations in the infant gut.<sup>6,7</sup> When oligosaccharides are consumed, the undigested portion serves as food for the intestinal microflora. Depending on the type of oligosaccharide, different bacterial populations are stimulated or suppressed. Probiotics are live bacteria and yeasts that benefit human

health, especially digestive health. Yogurt is one of the most familiar sources of probiotics—“good” bacteria that maintain a healthy balance in the human gut. Addition of prebiotics to probiotic foods has been demonstrated to have various benefits. In general, prebiotics promote the growth of the probiotic organism by providing the specific substrate for its fermentation.<sup>8</sup>

Characterization of different GOS has generally been done by fractionating the oligosaccharides (by yeast treatment, size-exclusion chromatography, hydrophilic interaction liquid chromatography) followed by a combination of analytical methods (methylation analysis followed by GC–MS, NMR spectroscopy, HPAE–PAD–MS, ESI–MS). Hernández-Hernández et al.<sup>9</sup> studied the glycosidic linkage types present in three commercial GOS samples (Vivinal, Bimuno®, and Yum-Yum™ GOS). They determined the linkages via MS fragmentation data; therefore, the anomeric configuration could be confirmed. All three GOS contained (1→6)-linked, (1→3)-linked, and (1→4)-linked β-D-Gal residues in varying abundance. The (1→2)-linked β-D-Gal residue was less common but was present in all three samples. Villaluenga et al.<sup>10</sup> reported a study on the determination of GOS present in 14 fermented milk samples. The HPAE-PAD method they developed and validated was applied to yogurts, yogurts containing bifidobacteria, and ready-to-drink yogurts containing *Lactobacillus casei*, providing comprehensive information about the total and individual content of GOS in commercial fermented milks. In a recent paper, Sims et al.<sup>11</sup> investigated the in vitro fermentation of prebiotic oligosaccharides by three probiotic bacteria—*Lactobacillus rhamnosus* HN001, *Lactobacillus acidophilus* NCFM, and *Bifidobacterium lactis* HN019. The oligosaccharides were separated on a Thermo Scientific™ Dionex™ CarboPac™ PA100 column and detected by PAD. Based on their HPAE-PAD profiling, they suggested combinations of pro- and prebiotics, *L. acidophilus*/FOS or nGOS and *L. rhamnosus*/BGO, in which the prebiotic might have the potential to maintain the viability of the bacteria in probiotic products and increase their persistence in the gastrointestinal tract.<sup>11</sup>

In this work, we demonstrate HPAE-PAD profiling of Bimuno GOS powder and Bimuno GOS syrup, and identify the proper labeling of GOS DPs in Bimuno GOS powder. Bimuno GOS powder was purchased online and used as a standard for the GOS profile. This product claims to contain 2.8 g of Bimuno GOS per 5.5 g of powder. It was later found out that it is not a good standard for the GOS profile due to the presence of other oligosaccharides. Bimuno GOS syrup was used to identify the correct GOS profile. Bimuno GOS syrup was kindly provided to us by a leading biotechnology company.

Separation of individual oligosaccharides in GOS was achieved on a Thermo Scientific™ Dionex™ CarboPac™ PA200 column. The Dionex CarboPac PA200 column was developed to provide higher-resolution separations of charged and neutral oligosaccharides than the Dionex CarboPac PA100 column, and is the recommended column for these applications. The Dionex CarboPac PA200 column is packed with a hydrophobic, polymeric, pellicular anion exchange resin that is stable over the range of pH 0–14. Oligosaccharide detection was by PAD with a gold working electrode, and therefore no sample derivatization was required.

## Experimental

### Equipment

- Thermo Scientific™ Dionex™ ICS-5000+ system, including:
  - SP Single Pump or DP Dual Pump
  - DC Detector/Chromatography Compartment
  - Dionex AS-AP Autosampler
  - ED Electrochemical Detector (without Cell, P/N 079830)
  - ED Cell with Reference Electrode and Spacer Block (P/N AAA-061756)
  - Gold on PTFE Disposable Electrode (P/N 066480)
  - pH-Ag/AgCl Reference Electrode (P/N 061879)
- Thermo Scientific™ Chromeleon™ Chromatography Data System (CDS) software was used for all data acquisition and processing.

## Consumables

- Thermo Scientific™ Nalgene™ Syringe Filters, PES, 0.2 µm (Fisher Scientific, P/N 09-740-61A)
- Air-Tite™ All-Plastic Norm-Ject™ Syringes, 5 mL, sterile (Fisher Scientific, P/N 14-817-28)
- Vial Kit, 10 mL Polypropylene with Caps and Septa (P/N 055058)
- Thermo Scientific™ Nalgene™ Rapid-Flow™ Sterile Disposable Filter Units with Nylon Membrane (1000 mL, 0.2 µm pore size, Fisher Scientific P/N 09-740-46)
- Amicon® Ultra-15 Centrifugal Filter Unit with Ultracel® -3 membrane (P/N UFC900396)

## Reagents and standards

- Deionized (DI) water, Type I reagent grade, 18 MΩ-cm resistivity or better
- Sodium hydroxide, 50% w/w (Fisher Scientific P/N SS254-500)
- Sodium acetate, anhydrous (NaOAc), electrochemical grade (P/N 059326)
- Bimuno GOS powder ([www.Bimuno.com](http://www.Bimuno.com))
- Amyloglucosidase powder (36000 U/g) (Megazyme, P/N E0AMGDFFD)

## IC conditions

System	Dionex ICS-5000+ HPIC System		
Column	Dionex CarboPac PA200 Guard, 3 × 50 mm (P/N 062895) Dionex CarboPac PA200 Analytical, 3 × 250 mm, (P/N 062896)		
Eluent	A) 100 mM sodium hydroxide (NaOH), B) 1 M sodium acetate (NaOAc), 100 mM NaOH		
Gradient	-5 min: 100 mM NaOH/50 mM NaOAc 0–45 min: 100 mM NaOH/ 50–150 mM NaOAc 45–55 min: 100 mM NaOH/500 mM NaOAc 55–60 min: 100 mM NaOH/50 mM NaOAc, curve 5		
Flow rate	0.5 mL/min		
Inj. volume	10 µL		
Inject mode	Push full		
Loop overfill factor	5		
Detection	Pulsed amperometry, Gold on PTFE Disposable Working Electrode (P/N 066480), Ag/AgCl reference		
Waveform	Time (s)	Potential (V)	Integration
	0.00	+0.1	
	0.20	+0.1	Begin
	0.40	+0.1	End
	0.41	-2.0	
	0.42	-2.0	
	0.43	+0.6	
	0.44	-0.1	
	0.50	-0.1	
System backpressure	~3100 psi		
Background	20–30 nC		
Noise	~50 pC peak-to-peak		
Run time	60 min		

## Preparation of solutions and reagents

### Eluent solutions

#### 100 mM sodium hydroxide

To make 0.1 M NaOH, add 5.2 mL of 50% (w/w) NaOH to 1 L of degassed DI water by removing the NaOH aliquot from the middle of the 50% solution where sodium carbonate is least likely to have formed. Do not pipet from the bottom where sodium carbonate precipitate may have fallen, and prepare eluent only from a bottle of 50% sodium hydroxide that still contains at least a third of its original volume. Place the tip of the pipette containing the aliquot of NaOH ~1 in. (2.54 cm) below the surface of the DI water and dispense the NaOH. If properly prepared without stirring, most of the concentrated sodium hydroxide will stay in the lower half of the container and the rate of carbon dioxide adsorption will be much lower than that of a homogenous solution. Seal the container after the sodium hydroxide transfer is complete. Immediately replace the cap on the 50% hydroxide bottle as well. Swirl to mix the contents of the tightly sealed container holding the 0.1 M hydroxide. Keep the eluent blanketed under helium or nitrogen at 34 to 55 kPa (5–8 psi) at all times and store for no more than ~1 week.

#### 1 M sodium acetate/100 mM sodium hydroxide

To make 1 L of 100 mM sodium hydroxide containing 1.0 M sodium acetate, dispense approximately 800 mL of DI water into a 1 L volumetric flask. Vacuum degas for approximately 5 min. Add a stir bar and begin stirring. Weigh 82.0 g anhydrous, crystalline sodium acetate. Add the solid acetate steadily to the briskly stirring water to avoid the formation of clumps, which are slow to dissolve. After the salt has dissolved, remove the stir bar with a magnetic retriever. Add DI water to the flask to bring the volume to the 1 L mark. Vacuum filter the solution through a 0.2 µm nylon filter. This can take some time because the filter may clog with insoluble material from the sodium acetate. Using a plastic volumetric pipette, measure 5.2 mL of 50% (w/w) sodium hydroxide solution from the middle of the bottle. Dispense the sodium hydroxide solution into the acetate solution ~1 in. (2.54 cm) under the surface of the acetate solution and then mix in the same manner as the 100 mM NaOH above. Keep the eluent blanketed under helium or nitrogen at 34 to 55 kPa (5–8 psi) at all times and store for no more than ~1 week. See Thermo Scientific Technical Note 71 for detailed information on eluent preparation for HPAE-PAD.<sup>12</sup>

## Standard solutions

### Bimuno GOS standard

Dissolve 0.2 g of Bimuno GOS in 100 mL DI water to make a 1000 mg/L stock standard. Store the stock standard at 4 °C. Using this stock standard, prepare working standards (20–400 mg/L) fresh daily. Pass the liquid through a Nalgene syringe filter before analysis. GOS standards must be prepared fresh daily to avoid the degradation of GOS and thus inconsistent results.

### Amyloglucosidase (270 U/mL)

Dissolve 75.2 mg powder in 10 mL of DI water. Aliquot solution to 2 mL vials and store at -20 °C.

## Sample preparation

### Sample

An organic infant formula (milk-based European infant formula)

- Weigh 1 g infant formula powder and dissolve in 50 mL DI water. Shake for 2–3 min.
- Transfer 12 mL to a 50 mL Amicon Ultra-15 centrifugal filter device and cap. Centrifuge for 60 min at 5000 rpm at 20 °C.
- Filter through a 0.2 µm filter and dilute the filtrate 20-fold with DI water before injection.

### Treatment with amyloglucosidase enzyme

Combine 1 mL of Bimuno GOS syrup/powder with 10 µL of amyloglucosidase enzyme solution in a 1.5 mL microcentrifuge tube. Heat the solution for 30 min at 40 °C. Vortex for 30 s and centrifuge for 1 min. Transfer the solution to the autosampler vial for analysis.

## Results and discussion

Oligosaccharides were separated using a Dionex CarboPac PA200 column (250 × 3 mm) in series with a Dionex CarboPac PA200 guard column (50 × 3 mm). A solution of Bimuno GOS was prepared and an aliquot (10 µL) of the solution was injected onto the column and eluted at 0.5 mL/min with a linear gradient of sodium acetate (50–150 mM in 45 min) in sodium hydroxide (100 mM NaOH). Figure 1 is the chromatographic profile of Bimuno GOS sample showing the separation of simple sugars (lactose, galactose, and glucose) and major oligosaccharides. GOS has been reported to show different chain lengths, ranging from DP2 to DP10 with a terminal glucose.<sup>13</sup> Coulier et al.<sup>4</sup> characterized a commercial GOS product (Vivinal GOS) using a combination of analytical techniques, including SEC, HPAE-PAD, and HPAE-MS, and reported it to contain oligosaccharides up to DP 7. For Bimuno GOS, we observed peaks in the 45 min separation window that we have tentatively identified as DP1–DP13.

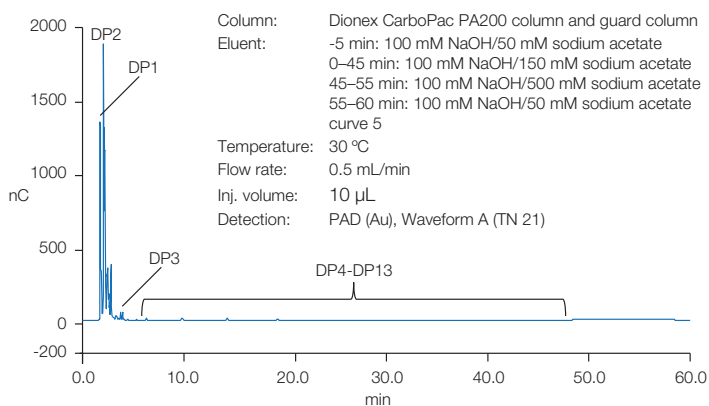


Figure 1. Chromatographic profile of Bimuno GOS sample

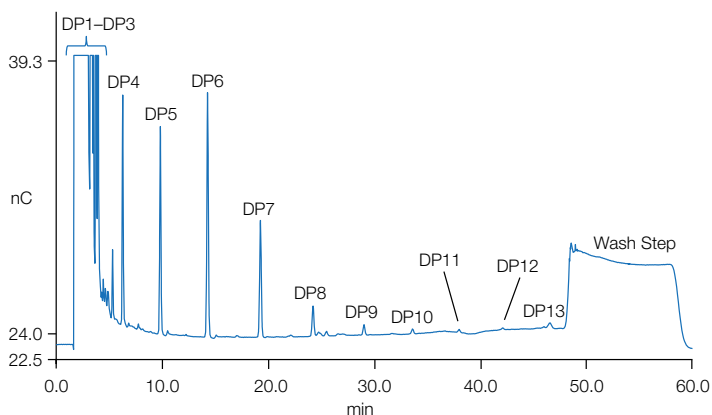


Figure 2. Magnified chromatographic profile of 100 mg/L Bimuno GOS sample enlarged to visualize DP4–DP13

The DP peak area distribution is skewed toward the smaller carbohydrates, DP1 and DP2, thus making it difficult to observe all the peaks in the same chromatogram. We enlarged one section of the Bimuno GOS chromatogram (Figure 2) to visualize DP4–DP13. The DP1 and DP2 peaks were identified based on the chromatogram of a mixture of glucose, galactose, and lactose. DP3 was identified by the chromatogram of maltotriose, a trisaccharide sugar consisting of three glucose molecules linked with  $\alpha$ -1,4 glycosidic bonds. Maltotriose elutes near the peak assigned as DP3. The assignment of the chromatographic peaks higher than DP3 was based on the generally accepted assumption that the retention time of a homologous series of carbohydrates increases as DP increases, and thus each successive peak represents a GOS that has one galactose more than the previous peak. Note that we did not confirm the identity of any of the DP3 to DP13 peaks.

Since the first publication of this application note we discovered that the peaks labeled as GOS DPs are maltodextrin DPs. As shown in Figure 3, the HPAE-PAD profile of Bimuno GOS powder matches a maltodextrin profile. Maltodextrin is typically composed of a mixture of chains that vary from three to 17 glucose units long, whereas GOS is a chain of galactose units that arise through consecutive transgalactosylation reactions, with a terminal glucose unit. GOS is usually produced by enzyme synthesis from lactose, and a product of this biosynthesis is a syrup that is finalized by a spray-drying process to produce a powder. Maltodextrin is frequently used as an additive in the spray-drying process to enhance the yields.<sup>14</sup> This is undoubtedly the reason we observe maltodextrin peaks in Bimuno GOS powder profile.

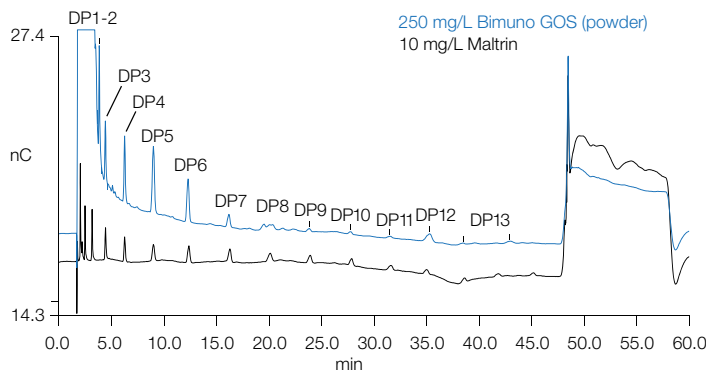


Figure 3. HPAE-PAD profile of 10 mg/L Maltrin and 250 mg/L Bimuno GOS (powder)

### Confirmation of maltodextrin peaks in Bimuno GOS powder profile

To confirm the presence of maltodextrin peaks in the Bimuno GOS profile, Bimuno GOS powder was treated with amyloglucosidase. Amyloglucosidase hydrolyzes  $\alpha$ 1,4-glucan bonds in polysaccharides with three or more  $\alpha$ 1,4-bound glucose units. Figure 4 shows the HPAE-PAD profile of enzyme treated Bimuno GOS powder along with untreated Bimuno GOS powder. As expected, maltodextrin DPs disappear from the Bimuno GOS powder profile, confirming maltodextrin DPs in the Bimuno GOS powder profile.

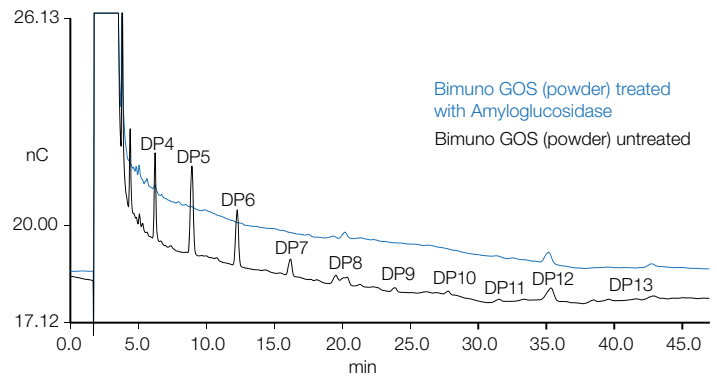


Figure 4. HPAE-PAD profile of 250 mg/L Bimuno GOS (powder) untreated and treated with amyloglucosidase

### HPAE-PAD profiling of Bimuno GOS syrup

Bimuno GOS syrup was kindly provided to us by a leading biotechnology company. We prepared and ran Bimuno GOS syrup under same eluent gradient conditions (100 mM NaOH/50–150mM NaOAc). Analysis of Bimuno GOS syrup by HPAE-PAD shows a complex pattern of peaks, which decrease in intensity with increasing retention time as can be seen in Figure 5. Coulier *et al.* observed a similar complex pattern for the Vivinal GOS profile. They also employed other analytical technologies such as SEC and HPAE-MS to demonstrate the complexity of GOS structures. Only a few peaks could be identified on the basis of reference compounds, that is, glucose, galactose, and lactose. Instead of a single peak, clusters of peaks were observed in the Bimuno GOS syrup profile, and these clusters of peaks are observed at regular intervals indicating increasing molecular weight. The retention time of a homologous series of carbohydrates increases as DP increases, and thus each successive peak represents a GOS that has one galactose more than the previous peak. Interestingly, it can also be seen from Figure 6 that there are similarities in the peak patterns between the different DP fractions, which indicate the presence of homologue oligosaccharide series. On the basis of the observed pattern of the clusters, we tentatively labeled them as clusters of DPs. To resolve the clusters, we used a shallower gradient 100 mM NaOH/(20–100) mM NaOAc (Figure 6). The clusters are more spread out and peaks within each cluster are better resolved, indicating the complexity of these GOS structures. To identify the structure of these complex GOS structures, a combination of analytical technologies are required.

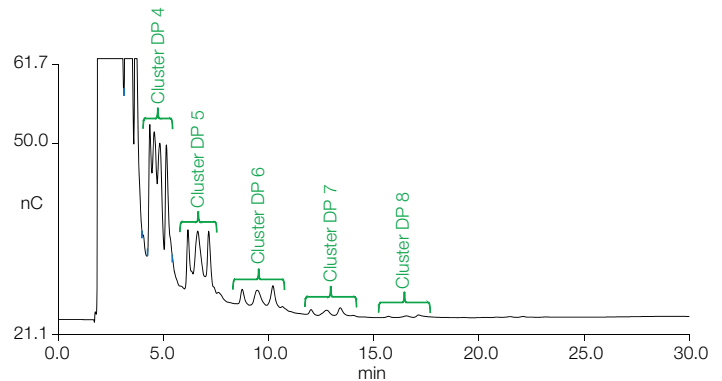


Figure 5. HPAE-PAD profile of 2000 mg/L Bimuno GOS (syrup)

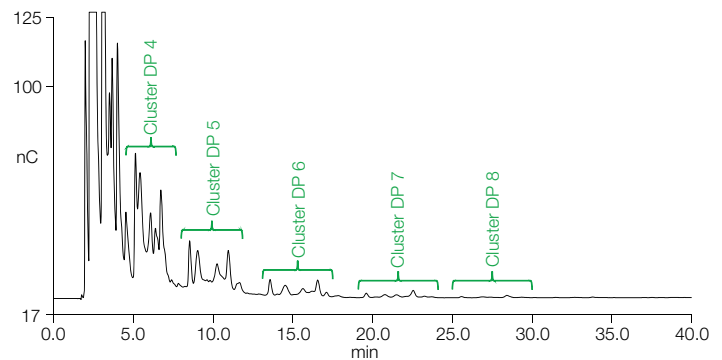


Figure 6. HPAE-PAD profile of 2000 mg/L Bimuno GOS (syrup) with a shallower elution gradient



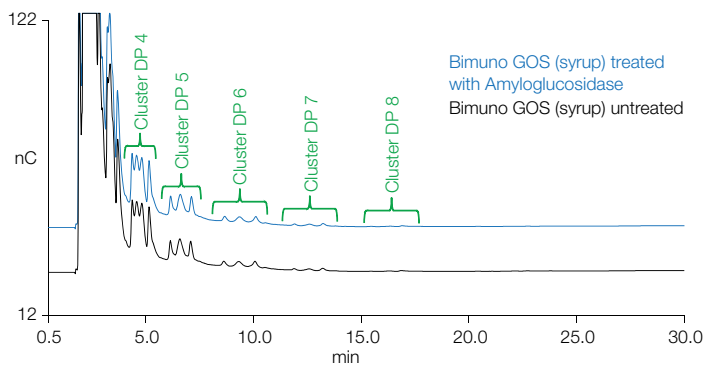


Figure 7. HPAE-PAD profile of 2000 mg/L Bimuno GOS (syrup) untreated and treated with amyloglucosidase

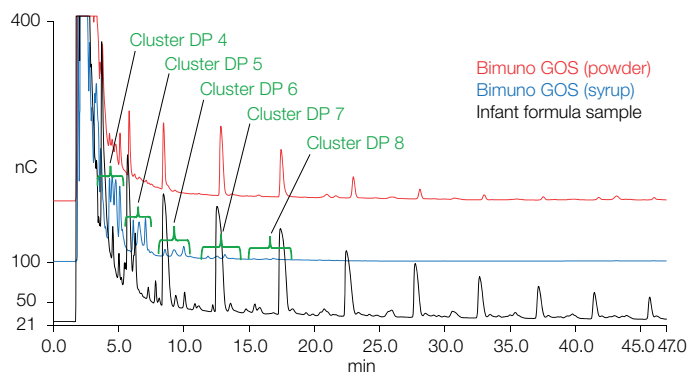


Figure 9. HPAE-PAD profile of GOS containing Infant formula sample along with 2000 mg/L Bimuno GOS (syrup) and 250 mg/L Bimuno GOS (powder)

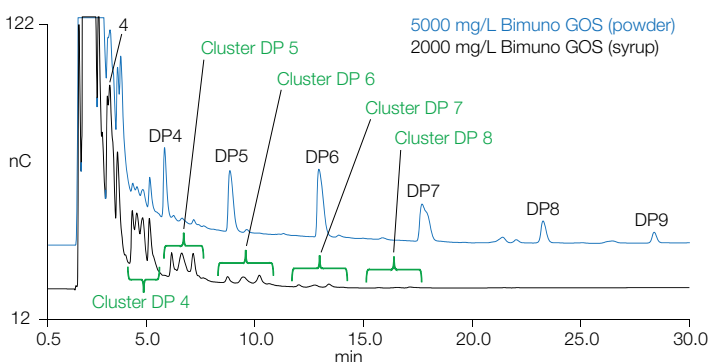


Figure 8. HPAE-PAD profile of 2000 mg/L Bimuno GOS (syrup) and 5000 mg/L Bimuno GOS (powder)

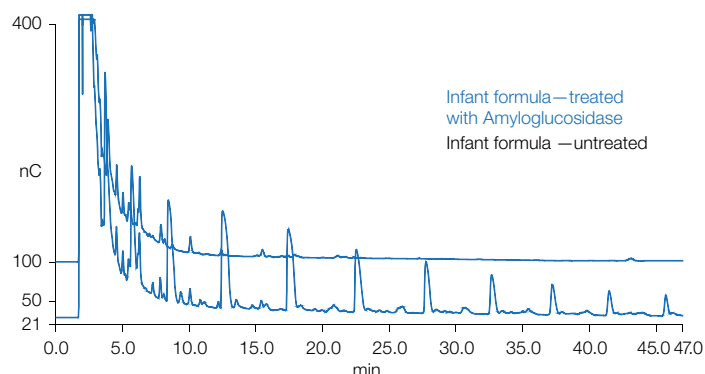


Figure 10. HPAE-PAD profile of Infant formula sample untreated and treated with amyloglucosidase

After treatment with amyloglucosidase enzyme, the Bimuno GOS syrup profile (Figure 7) remains the same, further suggesting that these clusters of peaks are a part of GOS and not maltodextrins.

These clusters of peaks were also observed in Bimuno GOS powder (Figure 8) at almost 20-fold higher concentration (~5000 mg/L). Due to the presence of maltodextrin peaks, they are not as distinct as is in Bimuno GOS syrup.

#### Column wash and equilibration between injections

For this method, perform a wash step after every injection to maintain column performance. The wash step consists of 10 min of 500 mM sodium acetate in 100 mM sodium hydroxide. This wash will ensure stable retention times and assist in maintaining a clean electrode. For good retention time reproducibility, the column must be equilibrated to the starting gradient conditions prior to each injection, and the

re-equilibration period should be tightly controlled. In all separations shown in this application note, the column set was re-equilibrated at initial conditions for 10 min, with the first minute used to return from the final gradient condition to the starting condition, prior to the next injection.

#### Determination of GOS in infant formula

A solution of infant formula was prepared and an aliquot (10  $\mu$ L) was injected on the same HPAE-PAD system using the same method as that for the Bimuno GOS. As shown in Figure 9, both maltodextrin peaks and GOS peak clusters (similar to the GOS syrup) are observed, but the peak pattern does not match the GOS syrup profile. This suggests this infant formula contains a different source of GOS. Upon treatment with amyloglucosidase, maltodextrin peaks disappear (Figure 10), confirming the presence of maltodextrin peaks in the infant formula powder sample.

## Conclusion

This work describes an HPAE-PAD method to profile GOS in commercial Bimuno GOS powder and Bimuno GOS syrup. The separation was achieved on a Dionex CarboPac PA200 column using a NaOH/NaOAc eluent. The GOS powder and an infant formula sample containing GOS both contained a high concentration of maltodextrins. We were able to profile the GOS in these samples by removing the interference from maltodextrins with amyglucosidase treatment. Due to the complex composition of GOS, the same GOS product added to the infant formulas should be used as the calibration standard to ensure method accuracy. This requirement can be met at the infant formula manufacturer where the GOS product and its specification information are available.

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