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. 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, cows, and wallabies. 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 β-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 β-D-Gal residue in the oligosaccharides, and fewer linkages such as (1→6)-linked β-D-Gal and (1→3)-linked β-D-Gal are observed. Depending on the enzymatic source used for their synthesis, the chemical structure of these oligosaccharides varies 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. 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.
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, HPAGE–PAD–MS, ESI–MS). Hernández-Hernández et al. 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. reported a study on the determination of GOS present in 14 fermented milk samples. The HPAGE-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. 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 HPAGE-PAD profiling, they suggested combinations of pre- and probiotics, 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.

In this work, HPAGE-PAD profiling was used to determine GOS content in probiotic samples and variation in prebiotics on addition of probiotics. We used Bimuno GOS as the prebiotic sample. Bimuno powder is a prebiotic food supplement containing uniquely patented B-GOS developed by scientists working at the University of Reading (UK). It is a mixture containing GOS, produced by the galactosyltransferase activity of Bifidobacterium bifidum NCIMB 41171 using lactose as the substrate. For probiotics, we used Yakult®, a well known probiotic dairy product. In this method, 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 high-resolution separations of charged and neutral oligosaccharides.

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™ Dionex™ 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)
**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.

### 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.

### 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.

**GOS Recovery**

- Weigh 1 g infant formula powder in a 50 mL centrifuge tube.
- Add ~60–120 mg Bimuno GOS powder to the centrifuge tube. Add 50 mL of DI water and shake for 2–3 min.
- Transfer 12 mL of above solution 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.
Addition of Probiotic Sample to Bimuno GOS
Mix 200 mg/L of Bimuno GOS sample with Yakult drink (10 mg/mL) in a 1:1 ratio. Incubate the mixture for 48 h in a water bath maintained at 37 °C. After incubation, transfer 12 mL to a 50 mL Amicon Ultra-15 centrifugal filter device and cap. Centrifuge for 30 min at 5000 rpm and 20 °C. Pass through a 0.2 μm filter before 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.13 Coulier et al.4 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. 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 α-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.

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.

Calibration of GOS
Bimuno GOS was used to prepare working standards. Figure 3 shows the chromatogram of the Bimuno GOS sample at five different concentrations (20, 50, 100, 200, and 400 mg/L). The calibration of GOS was based on individually calibrating each group of the major DP fractions. The marker peaks for DP1–DP3 were not chosen for the calibration due to the presence of interfering peaks.

Figure 1. Chromatographic profile of 100 mg/L Bimuno GOS sample.

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

Figure 3. Chromatographic profile of Bimuno GOS at 20, 50, 100, 200, and 400 mg/L. A 25% signal offset has been applied.
The concentration of each DP marker in the working standard is calculated using Equation 1.

\[
\% \text{ DP} = \left( \frac{\text{Area of individual DP}}{\text{sum of area of all DPs}} \right) \times 100 
\]

Concentration of DP 4 = \% DP (DP4) \times \text{total conc. (mg/L)}

DP4–DP 13 groups were calibrated individually by choosing a marker peak from each of the major DP groups, which was resolved from other peaks present in the sample chromatogram. As shown in Figure 4 and Table 1, the linearities of the calibration curves of the various marker peaks are excellent (\(r^2 \geq 0.999\)).

![Figure 4. Calibration curves of markers of DP4 through DP13.](image)

<table>
<thead>
<tr>
<th>Oligosaccharide</th>
<th>Coeff. of Determination (r^2)</th>
<th>C0 (Offset)</th>
<th>C1 (Slope)</th>
</tr>
</thead>
<tbody>
<tr>
<td>DP4</td>
<td>0.99946</td>
<td>0.0515</td>
<td>0.068</td>
</tr>
<tr>
<td>DP5</td>
<td>0.99978</td>
<td>0.0192</td>
<td>0.0705</td>
</tr>
<tr>
<td>DP6</td>
<td>0.99995</td>
<td>0.0044</td>
<td>0.071</td>
</tr>
<tr>
<td>DP7</td>
<td>0.99993</td>
<td>-0.005</td>
<td>0.0722</td>
</tr>
<tr>
<td>DP8</td>
<td>0.99993</td>
<td>-0.0009</td>
<td>0.0706</td>
</tr>
<tr>
<td>DP9</td>
<td>0.99991</td>
<td>-0.0006</td>
<td>0.0714</td>
</tr>
<tr>
<td>DP10</td>
<td>0.99987</td>
<td>0.0013</td>
<td>0.0699</td>
</tr>
<tr>
<td>DP11</td>
<td>0.99990</td>
<td>-0.0015</td>
<td>0.0733</td>
</tr>
<tr>
<td>DP12</td>
<td>0.99994</td>
<td>-0.0015</td>
<td>0.074</td>
</tr>
<tr>
<td>DP13</td>
<td>0.99994</td>
<td>0.0012</td>
<td>0.07</td>
</tr>
</tbody>
</table>

**Table 1.** Calibration parameters of DP1 through DP13.

**Determination of GOS in Infant Formula**

A solution of infant formula was prepared and an aliquot (10 µL) was injected on the same HPAE-PAD system using the same method as that for the Bimuno GOS. By comparing the areas of the two marker peaks in the infant formula sample and in the GOS ingredient, the GOS content was determined for the infant formula. The concentration of each marker from the major DPs was determined individually.
Equation 2 shows an example of the calculation for GOS concentration of an individual DP in the infant formula sample.

**Equation 2**.

\[
C_{dp4} (mg/g) = C_{cal} (mg/L) \times m \times M_{total} \times F / M_{powder}
\]

Where \( C_{dp4} \) is the concentration of the marker peaks, \( m \) is the dilution factor, which is 20 for the spiked samples; \( M_{total} \) is the total weight of the sample solution, which is the sum of 1 g infant formula powder and 50 mL DI water; \( F \) is the conversion factor to convert weight of the solution in grams into volume in liters; and \( M_{powder} \) is the weight of infant formula powder.

**Sample Recovery**

Method accuracy was evaluated by measuring GOS recoveries in spiked infant formula samples. GOS recoveries were determined based on calibrations using Bimuno GOS. Two spiked concentration levels in the European sourced infant formula sample spiked with Bimuno GOS were examined (Figure 5). The marker peak in the sample chromatogram was quantified based a calibration curve of peak area (nC*min) vs. concentration (mg/L) fit with a least squares regression. The % recovery data was obtained using the calculations in Equation 3.

**Table 2. Results of spike recovery for infant formula.**

<table>
<thead>
<tr>
<th></th>
<th>Spike Level 1</th>
<th>Spike Level 2</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Found (mg/g)</td>
<td>Added (mg/g)</td>
</tr>
<tr>
<td>DP4</td>
<td>18.2</td>
<td>6.38</td>
</tr>
<tr>
<td>DP5</td>
<td>9.17</td>
<td>7.20</td>
</tr>
<tr>
<td>DP6</td>
<td>11.5</td>
<td>10.0</td>
</tr>
<tr>
<td>DP7</td>
<td>8.08</td>
<td>5.33</td>
</tr>
<tr>
<td>DP8</td>
<td>2.75</td>
<td>1.50</td>
</tr>
<tr>
<td>DP9</td>
<td>1.17</td>
<td>0.54</td>
</tr>
<tr>
<td>DP10</td>
<td>0.61</td>
<td>0.28</td>
</tr>
<tr>
<td>DP11</td>
<td>0.44</td>
<td>0.14</td>
</tr>
<tr>
<td>DP12</td>
<td>0.19</td>
<td>0.08</td>
</tr>
<tr>
<td>DP13</td>
<td>3.32</td>
<td>0.46</td>
</tr>
</tbody>
</table>

Figure 5. Chromatographic profile of A) unspiked infant formula, B) spiked 1 infant formula, and C) spiked 2 infant formula. A 25% signal offset has been applied.

Equation 3.

\[
DP4 \text{ (Recovery \%)} = \frac{C_{\text{covered dp4}}}{C_{dp4} + C_{\text{added}}} \times \left( \frac{MB_{\text{Bimuno GOS}} \times \% DP \text{ fraction} \times P\%}{M_{\text{powder}}} \right)
\]

Where \( C_{\text{covered dp4}} \) and \( C_{dp4} \) calculated as shown above in Equation 2, \( C_{\text{added}} \) is the concentration of added GOS in mg/g, \( MB_{\text{Bimuno GOS}} \) is the weight of Bimuno GOS spiked to the infant formula powder, and \( P\% \) is the purity of the GOS in Bimuno GOS powder. As shown above, the concentrations were calculated for each DP (DP4–DP13) and then summed to obtain the total GOS content (Table 2). For the samples spiked and calibrated by Bimuno GOS, the recoveries were in the range of 96.1–110%.
Effect of Probiotic on Prebiotic

Among probiotics, the most important bacterial targets for selective stimulation are the indigenous bifidobacteria and lactobacilli. These microorganisms could be added to functional foods as probiotics. In this work we used Yakult drink, a product containing probiotics, to assess the effect on chain length distribution and amount of an added prebiotic, Bimuno GOS. Yakult is a probiotic dairy product made by fermenting skimmed milk with a special strain of the bacterium *Lactobacillus casei* strain Shirota. Yakult contains 6.5 billion counts of this special strain. HPAE-PAD chromatographic profiles allowed the comparison of prebiotic profile with and without incubation with probiotic-containing Yakult. As shown in Figure 6, six major peaks were observed in the chromatographic profile of Yakult sample. Peaks 1, 2, 3, and 5 have the same RTs as DP4, DP5, DP6, and DP13 in the chromatographic profile of the Bimuno GOS sample. Because the Yakult drink has other carbohydrate components such as corn dextrin, the five major peaks may be carbohydrates other than GOS. Table 3 lists the concentrations of the DPs calculated based on the individual calibration curves of the DP fractions of the Bimuno GOS (discussed above). With the exception of DP8, the concentrations for all the DPs decreased, indicating degradation of Bimuno GOS from the addition of probiotics. These results suggest that HPAE-PAD is a powerful tool for analysis of oligo- and polysaccharides in complex mixtures such as microbial and fecal cultures and can provide significant information about interactions among bacteria and prebiotic fibers. However, it is important to note that the conditions of our prebiotic/probiotic experiment are not optimum conditions for cell/microbial culture. These conditions were used to demonstrate the ability of HPAE-PAD to determine the changes in the chain length distribution and/or GOS content when mixed/incubated with bacteria. Experiments conducted under more realistic/typical culture conditions are discussed in the introduction.

![Figure 6. Bimuno GOS (a prebiotic sample) incubated with Yakult drink (a probiotic sample).](image)

<table>
<thead>
<tr>
<th>DP (mg/g)</th>
<th>DP4</th>
<th>DP5</th>
<th>DP6</th>
<th>DP7</th>
<th>DP8</th>
<th>DP9</th>
<th>DP10</th>
<th>DP11</th>
<th>DP12</th>
<th>DP13</th>
</tr>
</thead>
<tbody>
<tr>
<td>10 mg/mL Yakult drink</td>
<td>19.7</td>
<td>61.0</td>
<td>2.38</td>
<td>n.a.</td>
<td>1.69</td>
<td>n.a.</td>
<td>n.a.</td>
<td>n.a.</td>
<td>1.84</td>
<td></td>
</tr>
<tr>
<td>100 mg/L of Bimuno GOS</td>
<td>18.5</td>
<td>23.4</td>
<td>34.9</td>
<td>19.0</td>
<td>5.46</td>
<td>2.01</td>
<td>1.05</td>
<td>n.a.</td>
<td>0.70</td>
<td>0.43</td>
</tr>
<tr>
<td>1:1 Mixture of Bimuno GOS and Yakult</td>
<td>27.5</td>
<td>54.6</td>
<td>35.1</td>
<td>18.3</td>
<td>5.90</td>
<td>2.03</td>
<td>1.00</td>
<td>0.68</td>
<td>0.39</td>
<td>2.19</td>
</tr>
</tbody>
</table>

Table 3. Comparison of concentrations of individual DPs of Bimuno GOS profile with and without incubation with probiotic-containing Yakult.
Conclusion

This work describes an HPAE-PAD method to profile and determine GOS content in prebiotic samples. The separation was achieved on a Dionex CarboPac PA200 column using a NaOH/NaOAc eluent. The method is shown to have a linear range suited for handling high concentration samples with minimal sample treatment and good recoveries. The disposable gold working electrode provides consistently high detector response, assuring greater instrument-to-instrument and lab-to-lab reproducibility. The recoveries of GOS in infant formula samples were in the range of 96.1–110% using the Bimuno GOS powder. 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.

References