Profiling Fructooligosaccharide-containing Samples by HPAE-PAD

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Key Words

Dionex CarboPac PA200, prebiotics, probiotics, inulin, disposable electrodes

Goal

To develop a HPAE-PAD profiling method for the determination of fructooligosaccharide (FOS) content in prebiotic dietary supplements.

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.[1] Food ingredients that meet this definition are water-soluble carbohydrates such as fructooligosaccharides (FOS) and inulin, both part of the larger fructan family. These carbohydrates occur naturally in many foods of vegetable origin, such as onions, Jerusalem artichokes, asparagus, garlic, and chicory.

When oligosaccharides are consumed, the undigested portion serves as food for the intestinal microflora. Clinical studies have shown that administering FOS, galactosyl-oligosaccharides (GOS), or inulin can increase the number of these beneficial bacteria in the colon while simultaneously reducing the population of harmful bacteria.[2] FOS and inulin are resistant to direct metabolism in the stomach and reach the colon, where they are used by a select group of bacteria. Furthermore, when added to a food product, FOS and inulin can improve physical and structural properties of food such as hydration, oil-holding capacity, viscosity, texture, sensory characteristics, and shelf life.

Fructans are a class of dietary fiber. Both inulin and FOS are fructans, but they differ in polymerization. Inulin is a linear, highly polymerized fructan of degree of polymerization (DP) 11–60, consisting of a linear chain of fructose with β-(2 → 1) linkages and a terminal glucose unit. Inulins are a group of naturally occurring polysaccharides produced by many types of plants. When produced industrially, inulins are most often extracted from chicory. FOS has a DP of 2–9 and is produced from inulin by controlled hydrolysis. FOS and inulin differ by DP and molecular weight, depending on the source, harvest time, and processing conditions.[3]

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High-performance anion-exchange chromatography (HPAE), coupled with pulsed amperometric detection (PAD), enables complete, single-step separation of neutral and charged oligo- and polysaccharides differing by branch, linkage, and positional isomerism. HPAE-PAD is one of the most employed analytical techniques for the characterization of these molecules. Corradini et al.[4] demonstrated HPAE-PAD as a powerful tool to evaluate changes in chain length distribution of FOS and inulin fermented by bifidobacteria, providing significant information about their prebiotic capabilities. Addition of prebiotics to probiotic foods has been demonstrated to have various benefits: prebiotics promote the growth of the probiotic organism by providing the specific substrate for its fermentation.[5] Other authors reported that fructans enhanced the viability of probiotic bacteria in foods, such as yogurt[6] (i.e. they increased the shelf life of the product). HPAE-PAD had also been used to profile fructans in synbiotic fermented milk, which contains both prebiotics and probiotics. Borromei et al.[7] studied fermented milk samples during their shelf life. They analyzed the variations of the fructooligosaccaride profile and probiotic microorganisms count simultaneously for these milk samples to study the prebiotic and probiotic interactions.

In a recent paper, Sims et al.[8] 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 gastro-intestinal tract.

When investigating FOS and inulin for chains with high DP, the lack of commercial standards becomes an obvious problem. Peak identification is difficult and peak assignment is often based on a generally accepted assumption that retention of a homologous series of carbohydrates increases as the DP increases (i.e. each peak eluted represents a chain with one more unit than the previous peak).[9] This assumption is made because the larger the oligosaccharide, the greater the number of negatively charged functional groups.

In this work, the oligo- and polysaccharide distribution of three different inulin samples has been characterized by HPAE-PAD. In addition, HPAE-PAD profiling was demonstrated as a method to determine FOS content in prebiotic samples and variation in prebiotics on addition of probiotics. Inulin-FOS and chicory root inulin were used as prebiotic samples. For probiotics, we used Yakult® drink, a well known probiotic dairy product. In this method, separation of individual oligosaccharides in FOS was achieved on a Dionex CarboPac PA200 column. The Dionex CarboPac PA200 column was developed to provide high resolution separations of charged and neutral oligosaccharides and is the recommended column for these applications. The Dionex CarboPac PA200 column is packed with a hydrophobic, polymeric, pellicular anion exchange resin stable over a pH range of 0–14. Oligosaccharides were detected using PAD with a working gold electrode, therefore, no sample derivatization was required.

Equipment

- Thermo Scientific™ Dionex™ ICS-5000⁺ HPIC 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.

*The spacer block is needed when using the disposable working electrode. This method can also be executed with a conventional gold working electrode, though all the data presented in this application note were collected with disposable gold working electrodes.

Consumables

- Thermo Scientific™ Nalgene™ Syringe Filters, PES, 0.2 µm (Fisher Scientific, P/N 09-740-61A)
- AirTite™ 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, electrochemical grade (P/N 059326)
- Inulin, Chicory Root (Sigma Chemical)
- Inulin, Dahlia tubers (Sigma Chemical)
- Inulin-FOS powder (Jarrow's Formulas)

Experimental Conditions			
System	Dionex ICS-5000+ HPIC System		
Columns	Thermo Scientific™ Dionex™ CarboPac™ PA200 Guard, 3 × 50 mm (P/N 062895) Dionex CarboPac PA200 Analytical, 3 × 250 mm (P/N 062896)		
Eluent	A) 100 mM NaOH, B) 1 M NaOAc, 100 mM NaOH		
Gradient	-5 min: 100 mM NaOH/20 mM NaOAc, 0–15 min: 100 mM NaOH/20 mM NaOAc, 15–70 min 100 mM NaOH/20–450 mM NaOAc, 70–70.1 min: 100 mM NaOH/20 mM NaOAc, 70.1–75 min: 100 mM NaOH/20 mM NaOAc, Curve 5		
Flow Rate	0.5 mL/min		
Injection Volume	10 µL		
Inject Mode	Push full		
Loop Overfill Factor	5		
Detection	Pulsed amperometry, Gold on PTFE Disposable Electrode (6 pack) (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	~2900 psi		
Background	20–30 nC		
Noise	~50 pC/min peak-to-peak		
Run Time	75 min		

Preparation of Solutions and Reagents

Eluent Solutions

0.1 M sodium hydroxide (NaOH)

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 sodium hydroxide ~1 in (2.54 cm) below the surface of the DI water and dispense the sodium hydroxide. 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. Swirl to mix the contents of the tightly sealed container holding the 0.1 M sodium 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 (NaOAc)/0.1 M sodium hydroxide

To make 1 L of 0.1 M 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. Once 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 tip 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 0.1 M sodium hydroxide, 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.[10]

Standard Solutions

Inulin-FOS standard

Dissolve 0.1 g of Inulin-FOS in100 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.

Inulin Standard

Dissolve 0.1 g of Inulin (Chicory root and Dahlia tuber) 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.

Sample preparation

Mix 200 mg/L of Inulin-FOS sample with Yakult drink (10 mg/mL) 1:1. Incubate this mixture for 48 h in a water bath maintained at 37 °C. After incubation, transfer 12 mL of the solution 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

To find the best separation conditions that have good resolution at the beginning as well as at the end of a chromatogram and elute the higher DP fructans, various eluent combinations were tested. The goal was to achieve good resolution and reproducible retention times of FOS and inulin up to DP60. Baseline separation in a single run was obtained using the gradient profile described in the Conditions section, above. The separation was designed to be completed within 75 min. Using mild isocratic conditions at the beginning, glucose, fructose, and sucrose were resolved and then a linear gradient of sodium acetate (20–450 mM) was applied to separate fructans with a DP ranging from 3 (1-kestose) to 60. Thus, separation of both low and high molecular weight fructans was achieved.

The assignment of the chromatographic peaks with DP higher than 3 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 fructan with one fructose more than that represented by the previous peak. Moreover, the individual peaks were sharp and well resolved, similar to published separations.[9] Over the course of the analysis, the relative standard deviations of retention times of all eluted peaks (n = 6) ranged from 0.02–0.36%. Figure 1 shows a chromatographic profile of the Jarrow's inulin-FOS sample illustrating the separation of low molecular weight monosaccharides from high molecular weight FOS and fructans. Inulin-FOS is a prebiotic dietary supplement containing inulins and FOS that help in promoting probiotic bacterial growth. Figure 2 shows the chromatographic profile of a chicory inulin sample.

Column:	Dionex CarboPac PA200 + guard
Eluent:	-5 min: 100 mM NaOH/20 mM NaOAc, 0–15 min: 100 mM NaOH/20 mM NaOAc, 15–70 min 100 mM NaOH/20–450 mM NaOAc, 70–70.1 min: 100 mM NaOH/20 mM NaOAc, 70.1–75 min: 100 mM NaOH/20 mM NaOAc, curve 5
Temperature:	30 °C
Flow Rate:	0.5 mL/min
Inj Volume:	10 µL
Detection:	PAD(Au), Waveform A (TN 21)

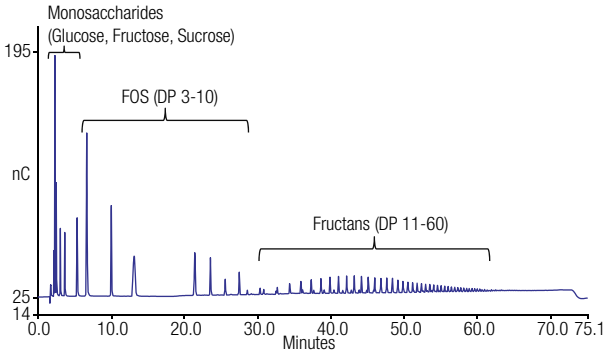

Figure 1. Chromatographic profile of Jarrow's inulin-FOS sample.

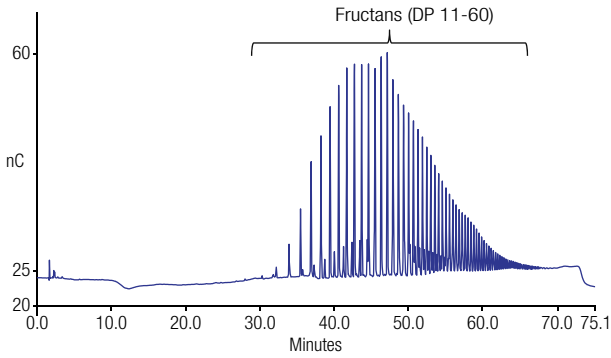

Figure 2. Chromatographic profile of inulin (chicory root) sample.

Calibration

Two prebiotic products (Jarrow's inulin-FOS and chicory inulin) were used to prepare working standards. Figure 3 shows the chromatogram of Jarrow's Inulin-FOS sample at five different concentrations (i.e. 20, 50, 100, 200, and 400 ppm). Five peaks (DP 22–26) were chosen as markers (Figure 4). The markers were chosen such that there was no closely eluting peak, though two of the markers have a shoulder. The concentration of these markers was determined by two methods. The first method is based on the % DP; the % DP for each marker was calculated by the following formula:

% DP = (Area of individual DP/ sum of area of all DP's) × 100

Concentration of marker 1 = % DP (marker 1) × total conc (mg/L)

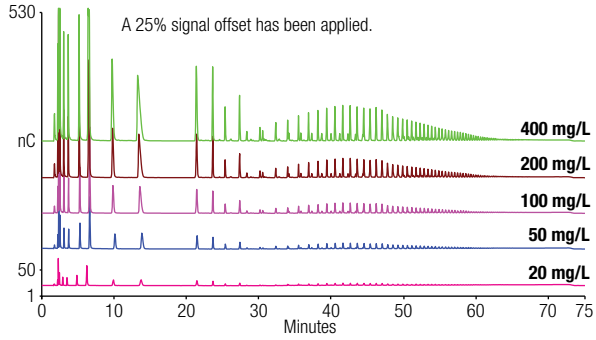

Figure 3. Chromatographic profile of Jarrow's inulin-FOS at 20, 50, 100, 200, and 400mg/L respectively.

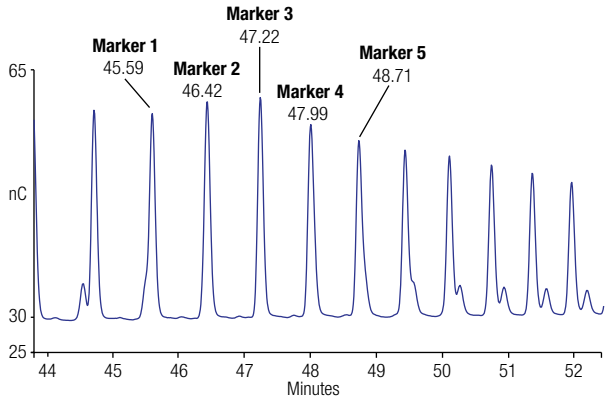

Figure 4. Chromatographic profile (zoomed in) of inulin- FOS sample showing the selected 5 marker peaks and their retention times.

In the second method, the markers are assigned the same concentration (i.e. 20, 50, 100, 200, and 400 ppm) and the calibration curve is plotted for all five markers. The calibration plots of peak area versus concentration were fit using linear regression functions, which yielded coefficients of determination (r^2) greater than 0.999. The calibration curves shown in Figure 5 for five markers are linear over the calibration range 20–400 mg/L.

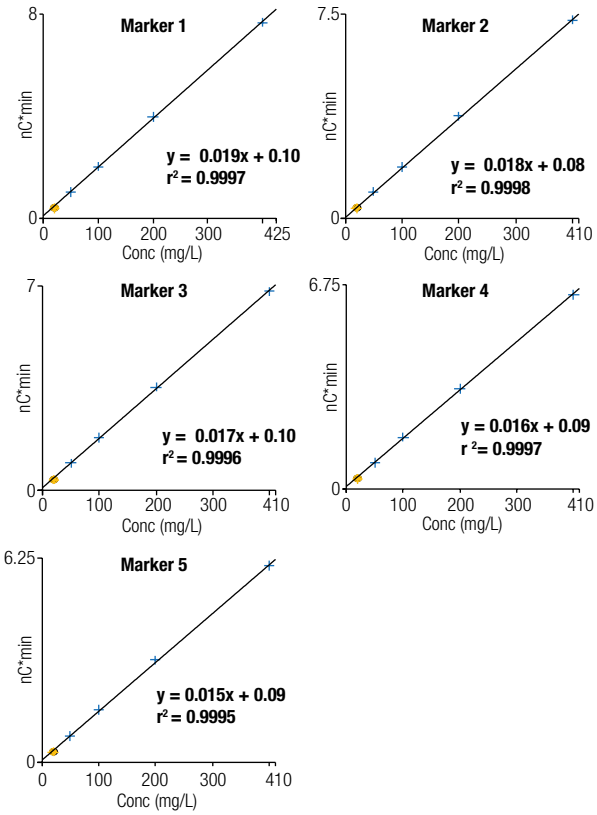

Figure 5. Calibration curves of markers 1, 2, 3, 4, and 5.

Sample Recovery

Method accuracy was evaluated by measuring recoveries in spiked Inulin-FOS samples. As noted earlier, there are no true standards available for these oligosaccharide samples. Thus we interchanged samples and standards according to the scheme shown below:

	Standard	Sample(s)	Recovery Results
Experiment 1	Jarrow's inulin-FOS	1. Inulin (chicory root)	Figure 6, Table 1
		2. Inulin (dahlia tubers)	Figure 7, Table 2
Experiment 2	Inulin (chicory root)	1. Jarrow's inulin-FOS	Figure 8, Table 3

Samples were spiked with standards at a level that was 50–100% of the amount determined in the original sample. Recoveries were calculated from the difference in response between the spiked and unspiked samples. The recovery percentages are within the range of 98–110%.

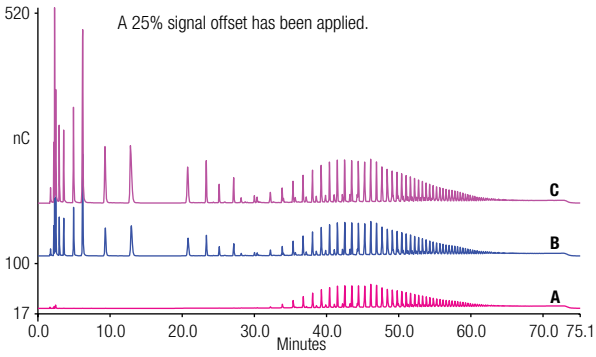

Figure 6. Chromatographic profile of A) unspiked inulin sample (chicory root) ; B) spiked inulin sample (with 50 mg/L inulin–FOS) and C) spiked inulin sample (with 100 mg/L inulin–FOS)

Table 1 . Results of spike recovery for inulin (chicory) in inulin FOS sample.

Calculation Method 1						
			50 mg/L Spiked		100 mg/L Spiked	
	Ret. Time (min)	Found (mg/L)	Spiked (mg/L)	Recovery (%)	Spiked (mg/L)	Recovery (%)
Marker 1	45.42	13.0	2.57	107	5.75	110
Marker 2	46.25	13.0	2.46	106	5.49	110
Marker 3	47.05	12.6	2.40	106	5.37	109
Marker 4	47.81	12.6	2.35	105	5.25	108
Marker 5	48.53	12.5	2.25	105	5.04	108

Calculation Method 2						
			50 mg/L Spiked		100 mg/L Spiked	
	Ret. Time (min)	Found (mg/L)	Spiked (mg/L)	Recovery (%)	Spiked (mg/L)	Recovery (%)
Marker 1	45.42	232	51.9	104	110	106
Marker 2	46.25	241	51.9	103	110	106
Marker 3	47.05	245	51.9	103	110	106
Marker 4	47.81	254	51.9	103	110	105
Marker 5	48.53	259	51.9	102	110	104

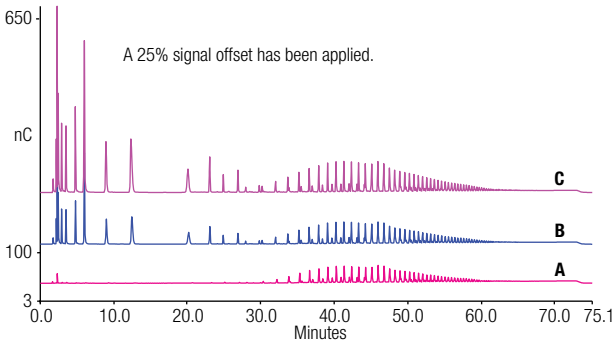

Figure 7. Chromatographic profile of A) unspiked inulin sample (dahlia tubers); B) spiked inulin sample (with 50 mg/L inulin–FOS) and C) spiked inulin sample (with 100 mg/L inulin–FOS)

Table 2 . Results of Spike Recovery for Inulin (dahlia) in inulin FOS sample.

Calculation Method 1						
			50 mg/L Spiked		100 mg/L Spiked	
	Ret. Time (min)	Found (mg/L)	Spiked (mg/L)	Recovery (%)	Spiked (mg/L)	Recovery (%)
Marker 1	45.42	8.92	2.57	102	5.75	109
Marker 2	46.25	8.86	2.46	105	5.49	110
Marker 3	47.05	8.27	2.40	104	5.37	109
Marker 4	47.81	8.13	2.35	101	5.25	110
Marker 5	48.53	7.87	2.25	103	5.04	109

Calculation Method 2						
			50 mg/L Spiked		100 mg/L Spiked	
	Ret. Time (min)	Found (mg/L)	Spiked (mg/L)	Recovery (%)	Spiked (mg/L)	Recovery (%)
Marker 1	45.42	163	49.8	99.3	99.6	109
Marker 2	46.25	166	49.8	102	99.6	108
Marker 3	47.05	164	49.8	101	99.6	108
Marker 4	47.81	166	49.8	98.2	99.6	109
Marker 5	48.53	167	49.8	100	99.6	108

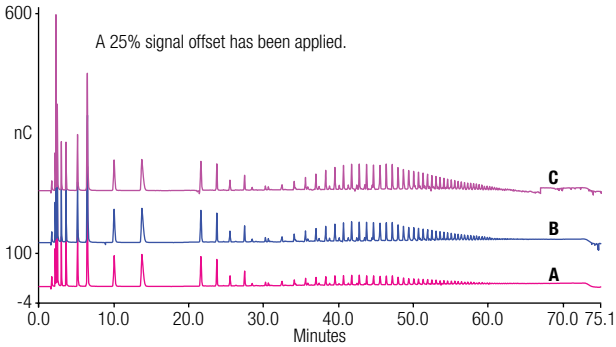

Figure 8. Chromatographic profile of A) unspiked inulin-FOS sample (Prebiotic); B) spiked inulin-FOS sample (with 50 mg/L chicory inulin) and C) spiked inulin sample (with 100 mg/L chicory inulin).

Table 3 . Results of spike recovery for inulin-FOS in inulin (chicory).

Calculation Method 1						
			50 mg/L Spiked		100 mg/L Spiked	
	Ret. Time (min)	Found (mg/L)	Spiked (mg/L)	Recovery (%)	Spiked (mg/L)	Recovery (%)
Marker 1	45.59	2.20	2.30	101	4.60	109
Marker 2	46.42	1.98	2.20	101	4.39	110
Marker 3	47.22	1.88	2.19	102	4.30	109
Marker 4	47.99	1.74	2.10	102	4.20	110
Marker 5	48.71	1.68	2.02	99.0	4.03	109

Calculation Method 2						
			50 mg/L Spiked		100 mg/L Spiked	
	Ret. Time (min)	Found (mg/L)	Spiked (mg/L)	Recovery (%)	Spiked (mg/L)	Recovery (%)
Marker 1	45.59	46.5	49.9	102	100	103
Marker 2	46.42	44.7	49.9	101	100	102
Marker 3	47.22	42.6	49.9	102	100	103
Marker 4	47.99	42.9	49.9	100	100	103
Marker 5	48.71	41.3	49.9	99.8	100	101

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, inulin-FOS. Yakult is a probiotic dairy product made by fermenting skimmed milk with a special strain of the bacterium *Lactobacillus casei Shirota*. It contains 6.5 billion counts of *Lactobacillus casei* strain Shirota. HPAE-PAD chromatographic profiles allowed the comparison of prebiotic profile with and without incubation with probiotic-containing Yakult. Figure 9 shows the results of this experiment. It demonstrates that the degradation of higher DP fructans is greater than the smaller FOS DP (≤ 10).

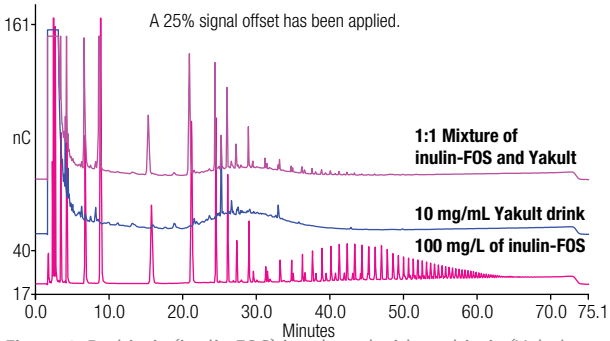

Figure 9. Prebiotic (inulin FOS) incubated with probiotic (Yakult drink)

These results suggest that HPAE-PAD is a powerful tool for profiling oligo- and polysaccharides in complex mixtures such as microbial and faecal cultures and can provide significant information about interactions among bacteria and prebiotic fibers. Moreover, it provides the possibility of evaluating an eventual synergic effect between prebiotics and probiotics and modulating the strains involved to optimize the composition to obtain the desired effect.

The information this method can supply may also be useful for defining the right health claims related to the quantified amount of FOS in the final product and not to the amount added. 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 only to demonstrate the ability of HPAE-PAD to determine the changes in the chain length distribution and/or FOS content when mixed/incubated with bacteria. Experiments conducted under more typical culture conditions are discussed in the introduction.

Conclusion

This work describes a HPAE-PAD method to profile FOS/fructans and determine FOS/fructan content in prebiotic samples. The Jarrow's inulin- FOS prebiotic sample was used as standard and spiking material for two inulins (chicory and dahlia tubers). The recoveries of fructans in samples were in the range of 98–110% . This work demonstrates that HPAE- PAD profiling is a powerful tool to evaluate the interactions of probiotics and prebiotics.

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