AUTHOR(S): BRUNT, K., SANDERS, P., SPICHTIG, V., ERNSTE-NOTA, V., SAWICKA, P., IWANOFF, K., VAN SOEST, J., KONG THOO LIN, P. and SEAN, A.

TITLE: Dual-laboratory validation of a method for the determination of fructans in infant formula and adult nutritionals.

YEAR: 2017

Publisher citation: BRUNT, K., SANDERS, P., SPICHTIG, V., ERNSTE-NOTA, V., SAWICKA, P., IWANOFF, K., VAN SOEST, J., KONG THOO LIN, P. and SEAN, A. 2017. Dual-laboratory validation of a method for the determination of fructans in infant formula and adult nutritionals. Journal of AOAC international [online], 100(3), pages 753-767. Available from: https://doi.org/10.5740/jaoacint.17-0007

OpenAIR citation: BRUNT, K., SANDERS, P., SPICHTIG, V., ERNSTE-NOTA, V., SAWICKA, P., IWANOFF, K., VAN SOEST, J., KONG THOO LIN, P. and SEAN, A. 2017. Dual-laboratory validation of a method for the determination of fructans in infant formula and adult nutritionals. Journal of AOAC international, 100(3), pages 753-767. Held on OpenAIR [online]. Available from: https://openair.rgu.ac.uk

Dual Lab Validation of a Method for the Determination of Fructans in Infant Formula and Adult Nutritionals

Kommer Brunt[1], Peter Sanders[2], Véronique Spichtig[3], Veronica Ernste-Nota[2], Paulina Sawicka[3,4,5], Kimberley Iwanoff[6], Jeroen Van Soest[2], Paul Kong Thoo Lin[4], Sean Austin[3]

1. Rotating Disc BV, 9753 HV Haren, The Netherlands
2. Eurofins Carbohydrate Competence Centre, 8440 AT Heerenveen, The Netherlands
3. Nestlé Research Centre, 1000 Lausanne, Switzerland
4. Robert Gordon University, Aberdeen, AB10 7AQ, United Kingdom
5. North East Scotland College, Aberdeen, AB25 1BN, United Kingdom
6. Nestlé Quality Assurance Centre, York, UK

Abstract

Until recently, only two AOAC official methods have been available for the analysis of fructans; AOAC 997.08 and AOAC 999.03. Both are based on the analysis of the fructan component monosaccharides, glucose and fructose, after hydrolysis. The two methods have some limitations due to the strategies employed for removing background interferences (such as sucrose, α-glucooligosaccharides, and free sugars). The method described in this paper has been developed to overcome those limitations. The method is largely based on AOAC 999.03, and uses combined enzymatic and solid phase extraction steps to remove the interfering components without impacting the final analytical result. The method has been validated in 2 laboratories on infant formula and adult nutritionals. Recoveries were in the range 86 – 119 %, with most being in the range 91-104%. Relative repeatabilities (RSD(r)) were in the range 0.7 – 2.6 %, with one exception when the fructan concentration was close to the LoQ resulting in an RSD(r) of 8.9 %. The performance is generally within the requirements outlined in the standard method performance requirements (AOAC SMPR 2014.002) which specifies recoveries in the range 90-110% and RSD(r) of below 6%.

Introduction

Inulin and fructooligosaccharides (FOS) are increasingly used as a health enhancing ingredients in a diverse range of food, feed and pet food products. Both inulin and FOS pass the stomach and small intestine unchanged and are fermented in the large intestine where they stimulate the growth and/or activity of bacteria like lactobacilli and bifidobacteria which may be beneficial to health (1,2).

Historically two different official AOAC methods have been available for the determination of the total fructan content in food products, being AOAC 997.08 (3) and AOAC 999.03 (4). The different underlying principles of the two methods results in each having different advantages and disadvantages. Prior knowledge of the sample composition (content of sugars, maltodextrins, starch) and of the fructan composition (presence of short chain oligo-fructose) is necessary to be able to select the best approach.

In the AOAC 997.08 method (3) first the free fructose and sucrose content have to be quantified chromatographically. In the next step, after enzymatic conversion of starch and maltodextrins, glucose is

again measured chromatographically. In the third step inulin/FOS and sucrose are completely converted into glucose and fructose and then the released monosaccharides are determined chromatographically. The fructan content is calculated by subtracting the glucose, sucrose and fructose contents measured in steps 1 and 2 from the total fructose and glucose content measured in step 3. This implies that large corrections have to be made for samples containing large quantities of fructose, glucose, sucrose, maltodextrin and/or starch. Subtraction of two large values in order to calculate much lower inulin/FOS values generally results in less precise data with large standard deviations. Nevertheless the method performs well when applied to products containing relatively low levels of interfering components.

The principle of the AOAC 999.03 method (4) differs from AOAC 997.08 (3) in that all monosaccharides present after combined α-glucanases and sucrase treatment are removed by converting them into alditols (via borohydride reduction). After enzymatic hydrolysis of the fructans, a colorimetric reducing sugar assay is then used to make the quantitative analysis. Fructans are thus accurately determined even in samples with high contents of monosaccharides, sucrose, maltodextrin and/or starch, using relatively simple and inexpensive equipment. However, there is a drawback to this method since the reducing end groups of the fructan chains that do not terminate with a glucose (often referred to as F_m type chains) are reduced into alditol end groups and so these escape the analysis resulting in low recoveries (for example, the theoretical recoveries of fructobiose (F2) is only 50%, for F3 67 %, for F4 75% etcetera). GFn molecules do not have a reducing end group, so these are recovered completely. FOS material prepared by depolymerization of inulin generally contain high amounts of F3 and F4, so total recovery can be below 80%. Although the method is not well suited to the analysis of samples containing FOS generated by partial hydrolysis of inulin, it is well suited to the analysis of long-chain fructans in a wide range of products and is quick and simple to apply.

Neither of the methods are optimal for the determination of fructans in infant formula or adult nutritionals. Therefore the stakeholder panel for infant formula and adult nutritionals (SPIFAN) issued a call for new methods to meet the standard method performance requirements (SMPR) defined in SMPR 2014.002 (5). After consideration by the Expert Review Panel (ERP) two methods, including the one described here, were considered to have acceptable performance.

AOAC 2016.06 (6,7) met those requirements. It is based on AOAC 999.03 (4) and a method published by Cuany et.al. (8) with a number of improvements. In AOAC 2016.06 (6, 7) a simplified sample preparation was introduced which reduces method turnaround time and concomitantly improves performance. The Cuany et.al method (8) required knowledge of the fructan type in the product to select appropriate correction factors (to correct for the “loss” of the terminal monomer of the fructan chains). Those correction factors are still required in AOAC 2016.06 (6,7), however a pre-analysis has been introduced to identify the fructan type, and thus the appropriate correction factor without the analyst needing (potentially confidential) recipe information.

The method described here has been designed to determine the fructan content without the need for correction factors, thus avoiding the need for pre-analysis (or knowledge of the fructan type in the product) and without interference from other components such as sucrose and free sugars. As in AOAC 999.03 (4), an enzyme mixture is used to hydrolyse sucrose and α-glucans to their constituent monosaccharides. Then, following the strategy of Cuany et.al. (8), a graphitized carbon solid phase extraction (SPE) column is used to eliminate the released glucose and fructose before the enzymatic hydrolysis of the fructans. The key difference is to avoid any borohydride treatment and thus avoid the

underestimation of F_m type fructans. The method was co-developed in two labs; Nestlé Research Centre (NRC) in Lausanne, Switzerland and the Carbohydrate Competence Centre of Eurofins (CCC) in Heerenveen, The Netherlands and single lab validations have been performed independently in both centres.

Experimental

Principle

Samples are reconstituted in water (if required) and further diluted until the concentration of fructan in solution is such that after hydrolysis the fructose and glucose concentration will be within the range covered by the standard curve. The diluted sample is treated with a mixture of sucrase and α-glucanases to hydrolyse sucrose and alpha-glucans to their constituent monosaccharides. The sample is passed through a solid phase extraction cartridge packed with graphitized carbon. Salts and monosaccharides pass through and are washed away, while the fructans are retained. Fructans are released from the column using an acetonitrile solution. The released fructans are hydrolysed with an inulinase mixture, and the released glucose and fructose are analysed by high performance anion-exchange chromatography with pulsed amperometric detection (HPAEC-PAD). The fructan content is calculated by summing the glucose and 0.9 × fructose content measured. In some matrices containing low amounts of fructans, a blank correction may be necessary and can be applied.

Materials

Samples from the SPIFAN II Single Laboratory Validation Kit were provided by Covance, and are listed in Table 1. In addition 2 infant formula from Nestlé, Vevey, Switerland were included in the SLV performed at NRC. Fructan ingredients used for spiking experiments were Orafti P95, Orafti HP (both from Beneo, Tienen, Belgium) and Nutraflora P95 (Ingredion, Westchester, IL, USA).

The samples were stored in the original package in a dry place, protected from light till the moment of use. According to the instructions for this SLV Kit and the AOAC SMPR 2014.002 (5), all powder products except SRM 1849a were reconstituted by dissolving 25 g of powder in 200 g of water. The SRM 1849a was weighed directly or it was reconstituted by dissolving 10 g in 90 g of water.

In this validation study three different standards of pure fructan ingredients have been used:

- Orafti HP, a long chain inulin ingredient
- Orafti P95, a hydrolyzed inulin ingredient consisting of both GFn and Fm constituents
- Nutraflora P95, a short chain fructo-oligosaccharide based on enzymatic sucrose elongation, mainly consisting of GF_2, GF_3, and GF_4 constituents

The purity of these standards was established by analysis according the AOAC 997.08 method (3).

Apparatus

(a) Analytical balance weighing to ± 0.1 mg (Mettler-Toledo, Greifensee, Switzerland)
(b) pH-meter, reading 0.1 pH (Metrohm, Herisau, Switzerland)
(c) Microcentrifuge tubes 2 mL (Eppendrof, Hamburg, Germany)

(d) Water bath at 80°C ± 1 °C (Thermo Fisher Scientific, Waltham, MS, USA)
(e) Water bath at 40°C ± 1 °C (Thermo Fisher Scientific, Waltham, MS, USA)
(f) Centrifuge for 2-mL microtubes able to operate at 10000 g (Eppendorf, Hamburg, Germany)
(g) Micropipettes with tips (0.1 to 1) mL (Socorex IsbA S.A., Ecublens, Switzerland)
(h) Vortex mixer (Scientific Industries, Bohemia, NY, USA)
(i) Single-use plastic pipettes 5 mL & 10 mL (Becton, Dickinson & Co, Franklin Lakes, NJ, USA)
(j) Single-use syringes 2 mL (Becton, Dickinson & Co, Franklin Lakes, NJ, USA)
(k) Graphitized carbon (100 mg) SPE columns (Sigma-Aldrich, St Louis, MO, USA)
(l) Membrane filter nylon, 0.2 µm, Ø 4.7 cm (Merck-Millipore, Darmstadt, Germany)
(m) Analytical column CarboPac PA20, (150 x 3 mm, 6.5 µm) or CarboPac PA1, (250 x 2 mm, 10 µm) with guard (50 x 2 mm, 10 µm) (Thermo Fisher Scientific, Waltham, MS, USA)
(n) Metal free liquid chromatograph (Thermo Fisher Scientific, Waltham, MS, USA) consisting of:-
- Gradient pump
- Autosampler
- Column compartment (able to maintain a temperature of 20°C (PA1) or 30°C (PA20))
- Electrochemical detector working in pulsed amperometric detection mode
- Isocratic pump for post-column delivery

Chemicals & Reagents

(a) Deionized Water (18 MΩ Milli-Q purified or equivalent)
(b) Maleic acid, puriss., >99 %, (Sigma-Aldrich, St Louis, MO, USA)
(c) Acetonitrile gradient grade for liquid chromatography (Merck-Millipore, Darmstadt, Germany)
(d) Acetic acid glacial 100 % anhydrous, GR for analysis (Merck-Millipore, Darmstadt, Germany)(Merck KGaA, Darmstadt, Germany)
(e) Potassium cyanohexaferrate(II)trihydrate [Optional] (Merck-Millipore, Darmstadt, Germany)
(f) Zinc acetate [Optional] (Merck-Millipore, Darmstadt, Germany)
(g) Trifluoroacetic acid (Sigma-Aldrich, St Louis, MO, USA)
(h) Hydrochloric acid (Merck-Millipore, Darmstadt, Germany)
(i) Sodium acetate, anhydrous (Sigma-Aldrich, St Louis, MO, USA)
(j) Sodium hydroxide 50 % (w/w) (J.T. Baker, Deventer, The Netherlands)
(k) Sodum hydroxide pellets (Merck-Millipore, Darmstadt, Germany)
(l) Sodium chloride (Merck-Millipore, Darmstadt, Germany)
(m) Sodium azide (Only when using PA1 column for HPAEC-PAD) (Sigma-Aldrich, St Louis, MO, USA)
(n) D-(-)-Fructose, >99 % (Sigma-Aldrich, St Louis, MO, USA)
(o) D-(+)-Glucose, ≥99.5 % (Sigma-Aldrich, St Louis, MO, USA)
(p) Chitobiose (Elicityl SA, Crolles, France)
(q) Mix of highly purified sucrase , β-amylase, pullulanase and maltase (from Fructan Assay Kit, K-FRUC from Megazyme, Bray, Ireland)
(r) Mix of highly purified exo-inulinase and endo-inulinase (from Fructan Assay Kit, K-FRUC from Megazyme, Bray, Ireland)

Preparation of Reagents

(a) *Sodium maleate buffer (100 mM, pH 6.5):* Into a large beaker (>1000 mL) weigh 11.6 g of maleic acid and dissolve with 900 mL of water (using magnetic stirrer). Adjust the pH to 6.5 with sodium hydroxide solution 1 M. Transfer the solution to a 1000 mL volumetric flask and make up to the mark with water.

(b) *Sodium acetate buffer (100 mM, pH 4.5):* Into a large beaker (>1000 mL) containing 900 mL of demineralised water, pipette 5.8 mL of glacial acetic acid. Adjust to pH 4.5 with sodium hydroxide solution 1 M. Transfer the solution to a 1000 mL volumetric flask and make up to the mark with water.

(c) *Chitobiose solution (600 µg/mL*): Into a 25 mL volumetric flask weigh 15 mg of chitobiose and make up to the mark with water.

(d) *Glucose stock solution (5 mg/mL):* Into a 25 mL volumetric flask weigh 125 mg of glucose and make up to the mark with water.

(e) *Fructose stock solution (10 mg/mL):* Into a 25 mL volumetric flask weigh 250 mg of fructose and make up to the mark with water.

(f) *Sodium hydroxide (1M):* dissolve 40 ± 1 g of sodium hydroxide pellets in 500 ml water in a 1000 ml volumetric flask. After cooling down to room temperature, make up to the mark with demineralized water and mix well.

(g) *Carrez I solution*: dissolve 106 g potassium hexacyanoferrate (II) trihydrate in 1000 ml demineralized water and store in a brown bottle [NB: Optional reagent]

(h) *Carrez II solution*: Dissolve in a 1000 ml volumetric flask 220 g zinc acetate in 900 ml demineralized water, add 29 ml glacial acetic acid. Make up to the mark with demineralized water and homogenize [NB: Optional reagent]

(i) *Sodium azide solution (0*.5%): Dissolve 1 g of sodium azide in 200 ml demineralized water [NB: Optional reagent, only needed for LC method on the PA1 column]

(j) *Sucrase / β-amylase / pullulanase / maltase:* Dissolve the contents of the vial containing powdered sucrase , β-amylase , pullulanase and maltase in 22.0 mL of sodium maleate buffer (100mM, pH 6.5). Mix well and divide into aliquots of 2.0 mL each and store frozen at -20°C in polypropylene tubes until use. NOTE: For the development and validation of this method, the pre-prepared enzyme mixture available in the Megazyme kit, K-FRUC, was used. When enzymes from another source are used it is imperative to ensure the enzyme mixture will completely hydrolyse any sucrose in the product without hydrolysing the fructan. This can be checked by performing an analysis with sucrose as an analyte and with a pure fructan as an analyte. No fructan should be measured when sucrose is analysed, and > 90% recovery should be achieved when a pure fructan is analysed.

(k) *Fructanase (exo-inulinase + endo-inulinase):* Dissolve the contents of the vial containing powdered exo-inulinase and endo-inulinase in 22.0 mL of sodium acetate buffer (100 mM, pH 4.5). Mix well and divide into aliquots of 2.0 mL each and store frozen at -20°C in polypropylene tubes until use. NOTE: For the development and validation of this method, the pre-prepared enzyme mixture available in the Megazyme kit, K-FRUC, was used. When enzymes from another source are used it is imperative to ensure the enzyme mixture employed will completely hydrolyse the fructan without hydrolysing any other glucose or

fructose containing oligo- or polysaccharide that may be present after treatment with the sucrase mixture above.

(l) *Wash solution for graphitized carbon column, TFA 0.1 % in acetonitrile 80 % (v/v):* Into a 100 mL volumetric flask, add 80 mL of acetonitrile (HPLC grade) and 100 µL of TFA. Make up to the mark with water.

(m) *Sodium chloride solution (1M) for graphitized carbon column:* Into a 100 mL volumetric flask weigh 5.8 g of sodium chloride and dissolve with 90 mL of demineralised water. Make up to the mark with water.

(n) *Elute solution for graphitized carbon column, TFA 0.05 % in acetonitrile 25 % (v/v):* Into a 100 mL volumetric flask, add 25 mL of acetonitrile (HPLC grade) and 50 µL of TFA. Make up to the mark with water.

Mobile Phase Preparation (Using CarboPac PA 20) performed at NRC

Eluent A for CarboPac PA20, sodium hydroxide solution (300 mM): Into a HPLC bottle, introduce 985 mL deionized water and degas with helium for 20 min. Add 15.6 mL of sodium hydroxide solution (50 %). Degas with helium for 20 min and keep under a blanket of helium until, and during, use.

Eluent B for CarboPac PA20, Milli-Q water: Into a HPLC bottle, introduce 2000 mL water and degas with helium for 20 min. Thereafter keep under a blanket of helium until, and during, use.

Eluent C for CarboPac PA20, sodium acetate (500mM) and sodium hydroxide (150 mM) solution: Into a 1000 mL volumetric flask, weigh 41.0 g of anhydrous sodium acetate and dissolve with 800 mL of water by mixing. Make up to the mark with water and filter on a 0.20 µm nylon membrane filter into an HPLC bottle. Degas with helium for 20 min then add (using a single-use plastic pipette) 7.8 mL of the 50 % (w/w) NaOH solution. Swirl gently to mix and sparge with helium for another 15 min. Thereafter keep under a blanket of helium until, and during, use.

Post-column addition reagent, sodium hydroxide (300 mM): Into a HPLC bottle, introduce 985 mL water and add 15.6 mL of the NaOH 50 % solution (using a single-use plastic pipette) swirl the solution gently to mix. Degas with helium for 20 min and keep under a blanket of helium until, and during, use.

Mobile Phase Preparation (Using CarboPac PA 1) performed at CCC

Eluent A for CarboPac PA1, sodium hydroxide solution (200 mM): weigh 3846 ± 5 g of deionized water in the eluent bottle and degas with helium for 20 min. Add 40 ml of sodium hydroxide solution (50%). Degas with helium for 20 min and keep under a blanket of helium until, and during, use.

Eluent B for CarboPac PA1, *Millipore Q water with sodium azide*: fill a 4 l eluent bottle with 3900 ml carbonate free Millipore-Q water. Add 100 ml 0.5% sodium azide solution. Degas with helium for 20 min and keep under a blanket of helium until, and during, use.

LC eluent C for CarboPac PA1, *sodium acetate solution (1 M)*: Into a 1000 mL volumetric flask, weigh 82.0 g of anhydrous sodium acetate and dissolve with 800 mL of water by mixing. Make up to the mark with

deionized water and filter on a 0.20 µm nylon membrane filter into an eluent bottle. Degas with helium for 20 min and keep under a blanket of helium until, and during, use.

LC post-column addition reagent, sodium hydroxide (300 mM): Into a HPLC bottle, introduce 985 mL water and add 15.6 mL of the NaOH 50 % solution (using a single-use plastic pipette) swirl the solution gently to mix. Degas with helium for 20 min and keep under a blanket of helium until, and during, use.

Preparation of Standards

Prepare a 6-level standard curve by diluting the glucose stock solution (5 mg/mL) and the fructose stock solution (10 mg/mL) as described in Table 2 using volumetric flasks made up to the final volume with deionised water.

Take the six solutions of standards and treat each one as follows: Into a microtube transfer 200 µL of standard solution and add 200 µL of water and 100 µL of chitobiose internal standard solution. Then transfer 400 µL of this solution to another tube and add 1200 µL of SPE elute solution. Then take 700 µL of that solution and add 300 µL of sodium acetate buffer. Mix well then centrifuge at 10000 × g. Transfer 900 µL of the supernatant in to a vial suitable for the instrument autosampler.

Sample Preparation

(a) For analysis of products on a ready to feed basis, reconstitute powder or liquid concentrates according to instructions. (For example, weigh 25 g of an infant formula powder in to a bottle and add water (200 g). Mix well at room temperature, and record the final weight.

(b) *Reconstituted products (as prepared above), or products which are sold as ready to feed (RTF),* are weighed (9 g, m) in to a 50 mL (V) volumetric flask and water (30mL) is added. Confirm the pH is between 5 – 9 (adapt if needed) and place in a water bath at 80°C with constant agitation for 20 min. After cooling make up to the mark with water (this is Solution A). Alternative dilutions schemes have also been applied (see Table 3)

(c) For analysis of powder products without prior reconstitution, weigh 1 g (m) of powder in to a 50 mL (V) volumetric flask. Add water (30 mL) *and confirm the pH is between 5 – 9 (adapt if needed).* Heat at 80 °C with constant agitation for 20 min. Cool to room temperature and complete to the mark with water (this is Solution A).

(d) The solutions prepared above are further diluted depending on the expected fructan content following the guidelines in the Table 3 and the resulting solution is “Solution B”.

(e) *Hydrolysis of sucrose and α-glucans:* Transfer 200 µL of Solution B in to a 1.5 mL microtube and add 100 µL of chitobiose solution (600 µg/mL) and 200 µL of the sucrose, maltase, amylase, pullulanase enzyme mixture. Mix well and incubate at 40°C for 90 min.

(f) *Optional Carrez Clarification:* (performed at CCC but not at NRC), add 10 µL Carrez I solution to the sample and mix well. Then add 10 µL of Carrez II solution and mix again. Centrifuge at 14’000 rpm for 10 min and use the supernatant for the next step.

(g) *Removal of monosaccharides (CCC Procedure)*

- Prepare the graphitized carbon SPE column as follows:-

- Flush with 3 × 400 µL of wash solution
- Flush with 3 × 400 µL of water
- Then perform the following steps under gravity (i.e without applying vacuum or positive pressure)
- Apply 400 µL of the enzyme treated solution.
- Wash with 1 × 400 µL of sodium chloride solution (1 M)
- Wash with 2 × 800 µL of sodium chloride solution (1 M)
- Wash with 5 × 800 µL of water
- Elute the fructans with using 5 × 400 µL of elute solution
- Mix eluates from the SPE cartridge well

(h) Removal of monosaccharides (NRC Procedure)

- Prepare the graphitized carbon SPE column as follows:-
- Flush with 3 × 400 µL of wash solution
- Flush with 3 × 400 µL of water
- Then perform the following steps under gravity (i.e without applying vacuum or positive pressure)
- Apply 400 µL of the enzyme treated solution.
- Wash with 2 × 1000 µL of sodium chloride solution (1 M)
- Wash with 4 × 1000 µL of water
- Elute the fructans in to a 2 mL microtube using 3 × 400 µL of elute solution
- Apply a little positive pressure to eliminate all solution from the column
- Mix eluate from the SPE cartridge well

(i) *Hydrolysis of Fructans (CCC procedure):* Transfer 1000 µL of the eluate from the SPE cartridge in to a microfuge tube and add 350 µL of sodium acetate buffer (100 mM, pH 4.5) and 100 µL of the inulinase mixture. Mix well and incubate at 40°C for 40 min.

(j) *Hydrolysis of Fructans (NRC procedure):* To the eluate from the SPE cartridge, add 300 µL of sodium acetate buffer (100 mM, pH 4.5). Transfer 700 µL of the solution in to a tube (marked sample) and add 100 µL of the inulinase mixture. In to a second microtube (marked blank) transfer 700 µL of the eluate and add 100 µL of sodium acetate buffer (100 mM, pH 4.5) (NB: The blank is only necessary for some matrices containing low amounts of fructans and may be skipped if it has already been established that it is not needed for a given matrix). For all tubes, mix well and incubate at 40°C for 40 min.

(k) After cooling, centrifuge at 10000 × g then transfer 700 µL of the supernatant in to a vial suitable for the instrument autosampler or pass the hydrolysate through a 0.2 µm syringe filter into the autosampler vial.

Chromatographic Conditions Using PA1 (CCC method)

The HPAEC-PAD system is equipped with the CarboPac PA1 guard (2 x 50 mm, 10 µm) and analytical (2 × 250 mm, 10 µm) columns or equivalent connected in series. The columns are held at 20°C and the injection volume is 20 µL. Sodium hydroxide (300mM) is added post-column (before the PAD detector) at a flow rate of 0.13 mL/min. Fructose and glucose are separated using the gradient described in

Table 4. Carbohydrates are detected by pulsed amperometry using the quadruple waveform described in Table 5.

Chromatographic Conditions Using PA20 (NRC method)

The HPAEC-PAD system is equipped with tha CarboPac PA20 (3 × 150 mm, 6.5 µm), or equivalent. The column is held at 30°C and the injection volume is 25µL. Sodium hydroxide (300mM) is added post-column (before the PAD detector) at a flow rate of 0.2 mL/min. Fructose and glucose are separated using the gradient described in Table 6. Carbohydrates are detected by pulsed amperometry using the quadruple waveform described in Table 5.

Calibration & Calculations

Use bracketed calibration, injecting 3 standards followed by 10 samples then 3 standards, etc. For example inject standards at levels 1,3 and 5 then 10 samples then standards at levels 2,4 and 6 then 10 samples, then standards 1,3,5, etc. For each analyte (glucose and fructose) use the instrument software to plot a six point standard curve of (Instrument response for analyte / Instrument response for internal standard) against the concentration of the analyte in the standard. Fit a quadratic curve to the data without forcing through zero. Use the calibration curve to calculate the glucose and fructose concentration in Solution B.

Calculate the concentration of fructan in the sample as follows:-

$C_G = C_{GB} \times D \times (V/m) \times 0.0001$

$C_F = C_{FB} \times D \times (V/m) \times 0.0001$

$TF = (C_F \times 0.9) + C_G$

Where,

TF = Total fructan in sample (in g/100 g)

C_G = Glucose released from fructan (in g/100 g)

C_{GB} = Glucose concentration in Solution B (in µg/mL)

C_F = Fructose released from fructan (in g/100 g)

C_{FB} = Fructose concentration in Solution B (in µg/mL)

D = Dilution factor between Solution A and solution B (from Table)

V = Total volume of Solution A (in mL)

m = Amount of sample weighed to prepare Solution A (in g)

0.0001 = factor to convert analyte concentration in solution (in µg/mL) to analyte concentration in sample (in g/100 g)

0.9 = factor to correct for uptake of water during fructan hydrolysis

For samples with low fructan content requiring the blank correction adapt the above equations as follows:-

$C_G = (C_{GB} – C_{G0}) \times D \times (V/m) \times 0.0001$

$C_F = (C_{FB} – C_{F0}) \times D \times (V/m) \times 0.0001$

Where

C_{G0} = Glucose concentration in Blank Solution B (in µg/mL)

C_{F0} = Fructose concentration in Blank Solution B (in µg/mL)

Validation design

Table 7 summarizes the main requirements described in SMPR 2014.002 (5) for the determination of fructans in infant formula and adult nutritionals. The single lab validations were designed to test the method against those requirements. Reproducibility could not be assessed in only 2 labs, however intermediate reproducibility was assessed, and provided a guide as to whether the reproducibility targets might be achievable.

Calibration Fit was assessed at NRC by injecting calibration solutions at 8 different concentrations (2 µg/mL – 300 µg/mL for glucose, 20 µg/mL – 1100 µg/mL for fructose) all containing the same amount of chitobiose internal standard. Each level was prepared in triplicate. The ratio of peak areas (analyte / chitobiose) were plotted against the analyte concentrations and a quadratic model was used to fit the data. The relative residuals were calculated and plotted against analyte concentration. At CCC the same approach was taken but using 12 different concentrations (0.051 – 21.78 µg/mL for glucose, 0.887 – 179 µg/mL for fructose).

Limits of Detection (LOD) and Quantification (LOQ) were assessed in slightly different ways in the 2 laboratories. At CCC an infant formula containing no fructans was spiked with a low level of fructan (just above the desired LOQ of 0.03 g/100g) and analysed 10 times (this was performed on 2 different days with a 3 month interval in between). The standard deviation of the results was multiplied by 3 to estimate the LOD and by 10 to estimate the LOQ. At NRC a different infant formula was selected. It was also a blank formula, but when analysed minor signals at the retention times of glucose and fructose could be observed. Those signals were treated as if they actually originated from fructan, and the amount of fructan they represented was measured 14 times (7 days in duplicate). The LOD and LOQ were then calculated by taking the mean fructan content and adding 3 times the standard deviation to estimate LOD and adding 10 times the standard deviation to estimate LOQ.

Repeatability (r) and intermediate reproducibility (iR) were assessed by analyzing samples (containing fructans) in duplicate on at least 6 different days. Excel, or the in-house statistical package Q-Stat, were used to calculate the SD(r) and SD(iR) using the following equations:-

SD(r)

$$SD(r) = \sqrt{\frac{\sum_{i=1}^{n} SD_i^2}{n}} = \sqrt{\frac{\sum_{i=1}^{n} (x_{i1} - x_{i2})^2}{2n}}$$

SD(iR) $$SD(iR) = \sqrt{SD^2(b) + \frac{1}{2} \times SD^2(r)}$$

Where:

n is the number of (single or duplicate) determinations
x_i is the individual result within the set of single determinations with i going from 1 to n ;
x_{i1} and x_{i2} are the two results within the set of duplicate determination with i going from 1 to n ;
SD(b) is the standard deviation between the means of duplicates

Recovery was assessed slightly differently in the 2 different laboratories. At NRC several different infant formula (containing no fructans) were spiked with 3 different levels of 3 different fructan ingredients. The fructan content of the ingredients was separately determined following the method AOAC 997.08 (3). The spiked samples were then analysed in duplicate on three different days, and the recovery calculated by comparing the measured amount with the theoretical (expected) amount. At CCC, six samples (containing fructans) were spiked with an additional 50 % or 150 % of the native fructan content (using the same 3 different fructan ingredients). The samples were also analysed in duplicate on three different days, and the recoveries were calculated by comparing the theoretical spike amount against the measured spike amount.

RESULTS

Method Development

The method essentially consists of 3 stages; (1) removal of sucrose and free sugars, (2) hydrolysis of fructan to release glucose and fructose, (3) analysis of the released glucose and fructose by HPAEC-PAD.

To optimize all parameters the final HPAEC-PAD method was first developed. In this case the two labs developed different solutions; NRC used a CarboPac PA20 column and CCC a CarboPac PA1 column (example chromatograms are shown in Figure 1). Each system has a dedicated elution gradient as described in the experimental section. In both cases the glucose and fructose are well separated from other sugars, including galactose which may be released from lactose if the inulinase used for fructan hydrolysis is insufficiently specific. The appearance of galactose in the chromatogram can thus be used as an indicator for this side activity. Both labs added sodium hydroxide solution post-column, prior to the PAD detector. The post-column addition of sodium hydroxide results in improved baseline stability and higher detector sensitivity. The PAD detector is an amperometric detector with a thin layer flow cell. Due to the impedance in the amperometric flow cell and the resulting ohmic drop in the potential of the working electrode, calibration curves of amperometric detectors deviate from linearity especially at higher analyte concentrations [9], therefore both labs employed quadratic calibration models.

The removal of sucrose is a particularly important part of the method, if not removed it will erroneously be included in the final fructan concentration. Sucrose can effectively and specifically be hydrolysed using a sucrase as described in AOAC 999.03 (4). However, after hydrolysis, instead of applying a sodium borohydride reduction to remove the released monosaccharides, we have employed solid phase extraction (SPE) on a graphitized carbon column. The starting conditions for the SPE were taken from the method described by Cuany et.al. (8), however using the conditions described it was noted that monosaccharides were not always 100% removed from some products. This problem was investigated

and it was found that when the sugars themselves were applied (or the hydrolysate of pure sucrose) then all sugars were removed. We concluded that in certain matrices there was a component of the sample retained in the SPE column, which in turn was retaining the monosaccharides (in particular glucose). To overcome this, a wash with sodium chloride solution was introduced. In most cases this was sufficient to disrupt the interaction and the monosaccharides were sufficiently removed. However, in a few instances, small amounts of glucose were still retained even after the sodium chloride wash. The amount retained is very low, thus it only significantly impacts the result when very low levels of fructan are being analysed. To address this issue we introduced the blank subtraction. To generate the blank, the sample is taken through the whole procedure but not treated with inulinase. Thus any erroneously trapped monosaccharides can be measured and the apparent fructan content of the blank subtracted from the result of the normally processed sample to achieve an accurate result.

The fructan hydrolysis employs the same enzymes as used in AOAC 999.03 (4). However, the sample is eluted from the SPE in a mixture of acetonitrile and dilute trifluoroacetic acid (TFA), which are not the optimal conditions for inulinase function. Previously (8) the samples were vacuum dried after SPE to remove the organic solvent and the TFA. However, the vacuum drying adds a considerable amount of time to the analysis. It was thus investigated if the enzymes could function in the presence of acetonitrile after pH adjustment. It was found to be the case, thus after SPE, all that is required is the addition of sufficient buffer to adjust the pH and then the enzymes function as normal. The amount of enzyme added was adapted to ensure complete hydrolysis of all fructan up to a content of 100% in powder products.

Lack of fit calibration

For both HPAEC-PAD systems (with the CarboPac PA 20 and the CarboPac PA1 column) good quadratic calibrations for both fructose and glucose were obtained with extended dynamic ranges and low relative residuals calculated from the differences in the predicted concentration and the actual concentration of the standards (Figure 2) The generally accepted criteria for a good calibration model is that the lack of fit for the standards should be less than 5% with the exception of the lowest standard. It is accepted that the lack of fit of one of the lowest standards may be higher. In both labs and for both analytes the residuals, at all but the lowest level, are less than 5 %, and at the lowest level they are below 10%.

Limit of detection and limit of quantitation (LOD/LOQ)

NRC established the LOD and LOQ by analyzing the blank sample Infant formula Powder Soy Based, lot number E10NWZC (Table 1, no 16) 14 times (7 days in duplicate). Minor signals present in the sample were quantified as fructan and the average fructan content (on a reconstituted basis) was determined to be 0.0025 g/100g with a standard deviation of 0.0016 g/100g. The limit of detection (LOD) and quantification (LOQ) were estimated as:
LOD = 0.0025 + (3 × 0.0016) = 0.0073 g/100g
LOQ = 0.0025 + (10 × 0.0016) = 0.018 g/100g
These results meet the requirements in the SMPR (5)

CCC used the sample Infant formula powder partially hydrolyzed milk based, lot number 410057652Z (Table 1, no 7) for the determination of the LOD and LOQ by spiking it with a low level of fructan (Orafti P95, 0.046 g/100g) and analyzing the sample 10 times on two different days.
Then the LOD and LOQ were calculated as:
LOD = 3 x SD
LOQ = 10 x SD
The results are summarized in Table 10 and also meet the requirements outlined in the SMPR (5). The established values of LOD and LOQ of both NRC and CCC are in good agreement meet the SMPR (5).

Precision

It was known that only a few samples in the SPIFAN sample kit contained fructan. In order to establish which samples contained fructan, both NRC and CCC analyzed the whole series of 19 samples independently from each other. In both labs six of the 19 samples were found to contain fructans, being:
- no 1, Child formula powder, lot 00847RF00
- no 9, Toddler formula powder milk based, lot 4052755861
- no 10, Infant formula powder milk based, lot 4044755861
- no 12, Child formula powder, lot 00866RF00
- no 14, Infant formula powder FOS/GOS based, lot 50350017W1
- no 19, Adult nutritional RTF high fat, lot 0729RF00

Those six samples were used in the precision study in both labs, an additional two samples (Nestlé Ref 1 and Ref 2) were included at NRC. The repeatability and intermediate reproducibility were assessed by analyzing all samples in duplicate on six different days, with the exception of Nestlé Ref 2 which was analysed in duplicate on 24 different days (Table 11).

The repeatability (RSD_r) achieved in both laboratories (Table 11) is well below the upper level of 6% defined in SMPR 2014.002 (5), with only one exception, sample 14, which had an RSD_r of 8.9 % at CCC. Sample 14 is the sample containing the lowest fructan content of about 0.03 g/100 g which is close to the limit of quantification, and thus the results may be expected to be more variable. The intermediate reproducibility (RSD_{iR}) achieved in both labs is in general below 8%, again with the exception of sample 14 analysed at CCC which was analysed with an RSD_{iR} of 14% which is almost certainly linked to the low concentration of analyte in that sample. No limits were defined for RSD_{iR} in SMPR 2014.002 (5), but the data suggests that achieving an RSD_R of below 12% (as defined in SMPR 2014.002) in a multi-lab trial (MLT) could be possible, although it may be expected that samples containing fructan levels close to the LOQ may have higher variability.

The analytical results and the established precision data of NRC and CCC agree very well with each other. There is no significant difference in the mean fructan contents of the samples (paired t-test ($\alpha = 0.05$)) but the RSD_{iR} values of the CCC are slightly higher than those of NRC (Table 11). This could be explained by the fact that CCC were able to introduce more variability in the execution of the intermediate reproducibility experiments than NRC; data at CCC were collected over the course of 4 months, while at NRC the data was mostly collected over the course of 1 month, data at CCC were acquired by 2 different people using 3 different instruments, while at NRC most of the precision data were generated by a single person on 2 different instruments.

Accuracy/Trueness

Primary indications of the accuracy of the method were obtained at CCC during the determination of LoD and LoQ, since this was performed by spiking low levels of fructans in to a blank matrix (Table 10). The fructan addition rate was 0.046 g/100 g on both days, thus the average recoveries were 95.6% on day 1 and 104% on day 2 which are well within the defined target range of 90 – 110 %.

For the full spike-recovery experiments, three different pure fructan ingredients were used; Orafti HP, Orafti P95 and Nutraflora P95. The ingredients were separately analyzed using the AOAC 997.08 official method (3) in order to confirm their purity.

At NRC six different blank matrices were spiked at three levels with the above mentioned three pure fructan ingredients on three different days in duplicate. All samples were initially analysed without using the blank subtraction (Table 12).

At the 2 higher spiking levels recoveries were in general very good (92 – 104 %) with one exception, the adult nutritional RTF high protein sample, for which the average recovery was only 86 %. At the lowest spike level (0.03 g/100g) which is equivalent to the LOQ specified in the SMPR (5), the recoveries were less good, varying from 101 – 151 %, with three matrices achieving the SMPR requirements (recoveries between 101 – 105 %) and three matrices being outside the requirements (recoveries between 114 – 151%). Because the spike level is very low, a small amount of interference can have a significant impact on the recovery. In order to correct for this interference the method using the blank subtraction was applied. Using the blank subtraction, recoveries on the samples with low spike levels are significantly improved to 95-119 % (Table 13), but still do not meet the SMPR in all cases (for two matrices the recovery exceeds 110%, at 117% and 119%). This improvement demonstrates the need for the blank subtraction for some samples, especially those containing low levels of fructans.

At CCC the six fructan-containing samples were over-spiked at about 50% and 150% level of the original fructan content determined in precision study. All samples, both non-spiked and spiked samples, were analysed without using the blank subtraction. The average recoveries (Table 14) were all within the target range of 90 – 110% defined in SMPR 2014.002 (5) with the exception of one sample (sample 9) which had an average recovery of 89% at the low spike level.

Method Specificity

There are potentially two different mechanisms that may cause interference in the method: (1) An interfering substance gives rise to a signal in the chromatogram which coelutes with the glucose or fructose peak leading to overestimation of the fructan content. (2) The presence of glucose and/or fructose containing carbohydrates (oligo- and polysaccharides) which are susceptible to hydrolysis by (side) activities of the fructanase used in last enzymatic hydrolysis step in the protocol. Both will give rise to a signal in the chromatogram and can lead to overestimation of the fructan content.

The specificity of the method was evaluated through a combination of the specificity of the enzymes employed for the sample preparation and the selectivity of the chromatographic system used for the final analysis. To investigate this a number of pure carbohydrate constituents, which may be present in infant formula and adult nutritionals, were subjected to the analysis (following the CCC procedure) in

order to investigate whether or not they falsely contribute to the fructan content. The following carbohydrates have been tested: resistant maltodextrin, soluble starch, isomaltulose, maltitol, sucrose, galactooligosaccharides and polydextrose , all with 0.5 gram sample weight. The results (Table 15) have been expressed as if the carbohydrate represented 12.5% of a reconstituted (or ready to feed) sample and also as if it represented 50% of a dry powder. The results (Table 15) have also been recorded with and without inclusion of the blank subtraction step.

The analytical results summarized in the column "Ingredient as 12.5% in RTF" show clearly that applying the standard protocol without blank correction none of the potentially interfering constituents, with the exception of polydextrose, gave rise to an erroneously high fructan content. All of the measured fructan levels were shown to be significantly lower than the LOQ. For the polydextrose the erroneously measured fructan content is at the level of the LOQ. However, when the blank correction is applied the interference is consistently below 0.01g/100g. The chromatographic profile of these two constituents on the CarboPac PA1 column (Figure 3) contains signals near the fructose and glucose peaks. Although the retention times differ somewhat from the calibration standards it is likely that they could interfere if present at very high concentration, however at typical usage levels in adult nutritionals and infant formula they should not represent a problem.

In the column "expressed in dry product" with "no blank correction" (Table 15), the GOS and the polydextrose result in the highest erroneous fructan contents (of 0.2 – 0.3 g/100g), the other ingredients produced results below 0.1 g/100g. Applying the blank correction resulted in a significant improvement, and all ingredients produced results below 0.1 g/100 g. These data indicate that those ingredients would have a negligible influence on the analysis of fructans in actual products.

Conclusions

The performance of the new method as established by two independent laboratories largely meets the requirements outlined in SMPR 2001.002 and the specificity and selectivity of the method are good. The good agreement of results between the two laboratories also indicates that the method is sufficiently robust to resist the minor changes in protocols between the two labs. The reduced number of chromatographic runs, and the elimination of the need for correction factors should be a significant advantage over the previous AOAC 997.08 and AOAC 999.03 methods.

References

1. Slavin, J. (2013) *Nutrients* **5**, 1417-1435 http://dx.doi.org/10.3390/nu5041417
2. Sherman, P.M. (2009) *J.Pediatr.* **155**, S61-S70 http://dx.doi.org/10.1016/j.jpeds.2009.08.022
3. *Official Methods of Analysis* (2016) 20th Ed., AOAC INTERNATIONAL, Rockville, MD, Method **997.08**
4. *Official Methods of Analysis* (2016) 20th Ed., AOAC INTERNATIONAL, Rockville, MD, Method **999.03**
5. *Official Methods of Analysis* (2016) 20th Ed., AOAC INTERNATIONAL, Rockville, MD, SMPR 2014.002. www.eoma.aoac.org
6. *Official Methods of Analysis* (2016) 20th Ed., AOAC INTERNATIONAL, Gaithersburg, MD, Method **2016.06**
7. Haselberger, P. & Jacobs, W.A. (2016) *J.AOAC Int.* **99**, 1576 – 1588 http://dx.doi.org/10.5740/jaoacint.16-0190
8. Cuany, D., Bénet, T. & Austin, S. (2010) *J. AOAC Int.* **93**, 202-212
9. Brunt, K., Bruins, C.H.P., & Doornbos, D.A. (1980) in *Electroanalysis in hygiene, environmental, clinical and pharmaceutical chemistry*, Smyth, W.F. (Ed), Elsevier Scientific Publishing Company, Amsterdam, The Netherlands, pp 327 - 336

Table 1: Contents of the SPIFAN SLV sample kit

No	Product description	Lot number		Country of origin
Placebo products				
1	Child formula powder	00847RF00	powder	United States
2	Infant element powder	00796RF	powder	United States
3	Adult Nutritional RTF, high protein	00821RF00	liquid	United States
4	Adult Nutritional RTF, high fat	00820RF00	liquid	United States
5	Infant formula RTF, milk based	EV4H2Q	liquid	United States
Fortified products				
6	SRM 1849a	CLC10-b	powder	United States
7	Infant formula powder partially hydrolyzed milk based	410057652Z	powder	United States
8	Infant formula powder partially hydrolyzed soy based	410457651Z	powder	United States
9	Toddler formula powder milk based	4052755861	powder	Ireland
10	Infant formula powder milk based	4044755861	powder	Ireland
11	Adult nutritional powder low fat	00859RF00	powder	United States
12	Child formula powder	00866RF00	powder	United States
13	Infant elemental powder	00795RF	powder	United States
14	Infant formula powder FOS/GOS based	50350017W1	powder	Switserland
15	Infant formula powder milk based	K16NTAV	powder	United States
16	Infant formula powder soy based	E10NWZC	powder	United States
17	Infant formula powder RTF milk based	EV4H2R	liquid	United States
18	Adult nutritional RTF high protein	00730RF00	liquid	United States
19	Adult nutritional RTF high fat	0729RF00	liquid	United States

Table 2: Dilution Scheme for the Preparation of the Standard Curve

Standard	Volume of fructose stock solution (µL)	Volume of glucose stock solution (µL)	Final Volume (mL)	Fructose Conc. (µg/mL)	Glucose Conc (µg/mL)
Level 1	200	40	100	20	2
Level 2	400	200	20	200	50
Level 3	800	400	20	400	100
Level 4	1200	600	20	600	150
Level 5	1600	800	20	800	200
Level 6	2000	1000	20	1000	250

Table 3: Possible Schemes for Sample Dilution Depending on Expected Fructan Content

Expected fructan content (g/100g)		Preparation of Solution A(1)			Dilution to Solution B		Dilution factor (D)
Powder	Ready-to-feed	Powder Weight (g)	RTF(2) Weight (g)	Final Volume (mL)	Volume of Solution A (mL)	Final Volume (mL)	
			Used at NRC				
< 4.5	< 0.5	1	9	50	No dilution	No dilution	1
4.5 - 9	0.5 - 1.0	1	9	50	5	10	2
9 - 27	1.0 – 3.0	1	9	50	5	25	5
27 - 36	3.0 – 4.0	1	9	50	5	50	10
36 - 45	4.0 – 5.0	1	9	50	5	100	20
			Used at CCC				
< 1	0.03 – 5.0	4	4	100	No dilution	No dilution	1
1 - 5	n/a	1	n/a	100	No dilution	No dilution	1
5 - 10	n/a	1	n/a	100	0.1	0.2	2
10 - 20	n/a	1	n/a	100	1	5	5
20 - 100	n/a	1	n/a	100	0.25	5	20

(1) Solution A is prepared either by diluting the indicated powder weight to the final volume or by diluting the indicated weight of RTF product to the final volume. (2) RTF = Ready to Feed product.

Table 4: HPAEC-PAD Gradient for PA1 or Equivalent

Time (min)	Flow (mL/min)	%A (200mM NaOH)	%B (Water)	%C (1 M NaOAc)
0.0	0.25	7.5	92.5	0.0
13.0	0.25	7.5	92.5	0.0
14.1	0.25	25.0	75.0	0.0
20.0	0.25	25.0	75.0	0.0
21.0	0.25	40.0	30.0	30.0
28.0	0.25	40.0	30.0	30.0
30.0	0.25	4.0	60.0	0.0
31.0	0.25	7.5	92.5	0.0
43.0	0.25	7.5	92.5	0.0

Table 5: Quadruple Waveform for Carbohydrate Detection

Time (s)	Voltage (V)	Gain Region
0.00	+ 0.10	Off
0.20	+ 0.10	On
0.40	+ 0.10	Off
0.41	- 2.00	Off
0.42	- 2.00	Off
0.43	+ 0.60	Off
0.44	- 0.10	Off
0.50	-0.10	Off

Table 6: HPAEC-PAD Gradient for PA20 or Equivalent

Time (min)	Flow (mL/min)	%A (300mM NaOH)	%B (Water)	%C (500mM NaOAc + 150 mM NaOH)
0.0	0.5	2	98	0
17.0	0.5	2	98	0
17.1	0.5	0	0	100
22.0	0.5	0	0	100
22.1	0.5	100	0	0
27.0	0.5	100	0	0
27.1	0.5	2	98	0
33.0	0.5	2	98	0

Table 7: Standard Method Performance Requirements for the Determination of Fructans in Infant Formula & Adult Nutritionals

Minimal requirements for the FOS determination	
Analytical range	0.03 – 5.0 g/100 g
LOQ	≤ 0.03 g/100 g
Repeatability (%RSD_r)	≤ 6 %
Reproducibility (%RSD_R)	≤ 12 %
Recovery	90 – 110 %

From SMPR 2014.002 (5)]. Concentrations apply to the product as consumed (i.e. on reconstituted powders or concentrates or "as-is" on ready to feed products).

Table 8: Design of spike-recovery experiment at NRC

No	Sample	Level 0 (0 g/100g)	Level 1 (0.03 g/100g)	Level 2 (2 g/100g)	Level 3 (5.0 g/100g)
15	Infant Formula Powder Milk Based	none	Orafti P95	Orafti HP	NutraFlora P95
16	Infant Formula Powder Soy Based	none	Orafti HP	NutraFlora P95	Orafti P95
18	Adult Nutritional RTF High Protein	none	NutraFlora P95	Orafti P95	Orafti HP
11	Adult Nutritional Powder Low Fat	none	Orafti P95	Orafti HP	NutraFlora P95
7	Infant Formula Powder Partially Hydrolyzed Milk Based	none	Orafti HP	NutraFlora P95	Orafti P95
13	Infant Elemantal Powder	none	NutraFlora P95	Orafti P95	Orafti HP

Table 9: Design of Spike-Recovery Experiment at CCC

No	Sample Description	Spike Level	Day 1 Orafti P95 Spike (g/100g)	Day 2 Nutraflora P95 Spike (g/100g)	Day 3 Orafti HP Spike (g/100g)
1	Child formula powder	Low	0.17	0.17	0.19
		High	0.49	0.50	0.53
9	Toddler formula powder milk based	Low	0.17	0.17	0.19
		High	0.49	0.50	0.53
10	Infant formula powder milk based	Low	0.17	0.17	0.19
		High	0.49	0.50	0.53
12	Child formula powder	Low	0.17	0.17	0.19
		High	0.49	0.50	0.53
14	Infant formula powder FOS/GOS based	Low	0.017	0.017	0.019
		High	0.049	0.050	0.053
19	Adult nutritional RTF high fat	Low	0.17	0.18	0.19
		High	0.49	0.50	0.53

Table 10: Determination of Limits of Detection & Quantification at CCC

	Day 1[1]		Day 2[1]	
	Fructan (g/100 g)	Meets SMPR requirements	Fructan (g/100 g)	Meet SMPR requirements
Average[2]	0.044	n/a	0.048	n/a
SD[2,3]	0.002	n/a	0.003	n/a
LOD = 3 x SD	0.005	Yes	0.009	Yes
LOQ = 10 x SD	0.016	Yes	0.030	Yes

1 Day 1 and Day 2 were separated by 3 months, 2 average and standard deviation calculated on 10 replicates measured on a single day, 3 SD = standard deviation

Table 11: Summary of Results from the Precision Study

	sample	n (days × replicates)		Mean fructan content (g/100 g)		RSD_r (%)		Meets SMPR 2014.002	RSD_{iR} (%)	
		NRC	CCC	NRC	CCC	NRC	CCC	target	NRC	CCC
1	Child formula powder	6 × 2	6 × 2	0.27	0.29[*)]	0.8	1.4[*)]	yes/yes[*)]	1.1	7.8[*)]
9	Toddler formula powder milk based	6 × 2	6 × 2	0. 21	0.22	2.0	1.6	yes/yes	2.4	6.1
10	Infant formula powder milk based	6 × 2	6 × 2	0.28	0.26	2.4	1.2	yes/yes	2.8	6.9
12	Child formula powder	6 × 2	6 × 2	0.28	0.28	1.2	1.1	yes/yes	1.1	6.1
14	Infant formula powder FOS/GOS based	6 × 2	6 × 2	0.03	0.04	0.9	8.9[**)]	yes/no[**)]	2.5	14.2[**)]
19	Adult nutritional RTF high fat	6 × 2	6 × 2	0.48	0.51	1.8	2.6	yes/yes	1.6	4.2
	Nestle Ref 1	6 × 2	n/a	2.9	n/a	1.1	n/a	yes	1.8	n/a
	Nestle Ref 2	24 × 2	n/a	0.39	n/a	3.3	n/a	yes	4.3	n/a

[*)] one Grubb's outlier in one of the duplicates; results therefore based on a set of 5 duplicates instead of 6 duplicates

[**)] fructan content close to LOQ resulting in somewhat increased RSD_r and RSD_{iR}

Table 12: Spike-Recovery Results At NRC

	Sample	Recovery Level 1			Level 2			Level 3		
		Spike (g/100g)	Rec (%)	RSD (%)	Spike (g/100g)	Rec (%)	RSD (%)	Spike (g/100g)	Rec (%)	RSD (%)
7	Infant Formula Powder Partially Hydrolyzed Milk Based	0.031	122	7.3	2.00	103	2.5	5.01	92.0	2.2
11	Adult Nutritional Powder Low Fat	0.031	102	5.1	1.99	102	2.0	5.02	102	1.6
13	Infant Elemental Powder	0.030	105	5.2	2.02	95.7	1.8	5.00	95.5	6.1
15	Infant Formula Powder Milk Based	0.031	101	5.0	2.00	99.7	2.2	5.02	98.2	2.4
16	Infant Formula Powder Soy Based	0.030	114	3.0	2.02	104	4.2	5.02	93.6	2.8
18	Adult Nutritional RTF High Protein	0.030	151	11	1.99	95.5	2.2	4.95	86.0	3.8

Table 13: Spike-Recoveries when Blank Subtraction was Applied for Low Spike Level at NRC

Sample		Recovery		
no	**description**	Spike (g/100g)	Rec (%)	RSD (%)
7	Infant Formula Powder Partially Hydrolyzed Milk Based	0.031	117	12
11	Adult Nutritional Powder Low Fat	0.031	95.6	7.4
13	Infant Elemantal Powder	0.030	96.0	5.6
15	Infant Formula Powder Milk Based	0.031	95.4	2.6
16	Infant Formula Powder Soy Based	0.030	104	11
18	Adult Nutritional RTF High Protein	0.030	119*	7.1

* sample analysed on 4 days in duplicate, all others on 3 days in duplicate.

Table 14: Spike-Recovery Results At CCC

	sample	addition spiked level (g/100 g)	average recovery (%)	SD_{Rec}
1	Child formula powder	Low (0.17 – 0.19)	90.8	6.3
		High (0.49 – 0.53)	95.7	3.8
9	Toddler formula powder milk based	Low (0.17 – 0.191)	89.0	4.7
		High (0.49 – 0.53)	93.2	1.2
10	Infant formula powder milk based	Low (0.17 – 0.19)	94.1	2.1
		High (0.49 – 0.53)	94.6	2.9
12	Child formula powder	Low (0.17 – 0.19)	91.0	4.9
		High (0.49 – 0.53)	101.5	7.1
14	Infant formula powder FOS/GOS based	Low(0.017 – 0.019)[*)]	92.8[**)]	6.3
		High (0.049 – 0.053)	92.2	5.5
19	Adult nutritional RTF high fat	Low (0.17 – 0.19)	94.8	9.2
		High (0.49 – 0.53)	95.3	4.5
	average recovery		**93.7**	

[*)] Spiked level is less than the LOQ concentration of 0.03 % m/m

[**)] one (Grubb's) outlier recovery value (56.8%) rejected

Table 15: Results of Specificity Experiments

Ingredient	fructan content (g/100 g)			
	Ingredient as 12.5% in RTF		Ingredient as 100% of dry product	
	no blank correction	with blank correction	no blank correction	with blank correction
Resistant dextrin	0.004	0.002	0.035	0.014
Soluble starch	0.010	0.009	0.077	0.069
Isomaltulose	0.005	0.004	0.037	0.033
Maltitol	0.002	0.001	0.015	0.007
Sucrose	0.006	0.005	0.049	0.041
Galactooligosaccharides	0.023	0.002	0.182	0.017
Polydextrose	0.034	0.007	0.271	0.058

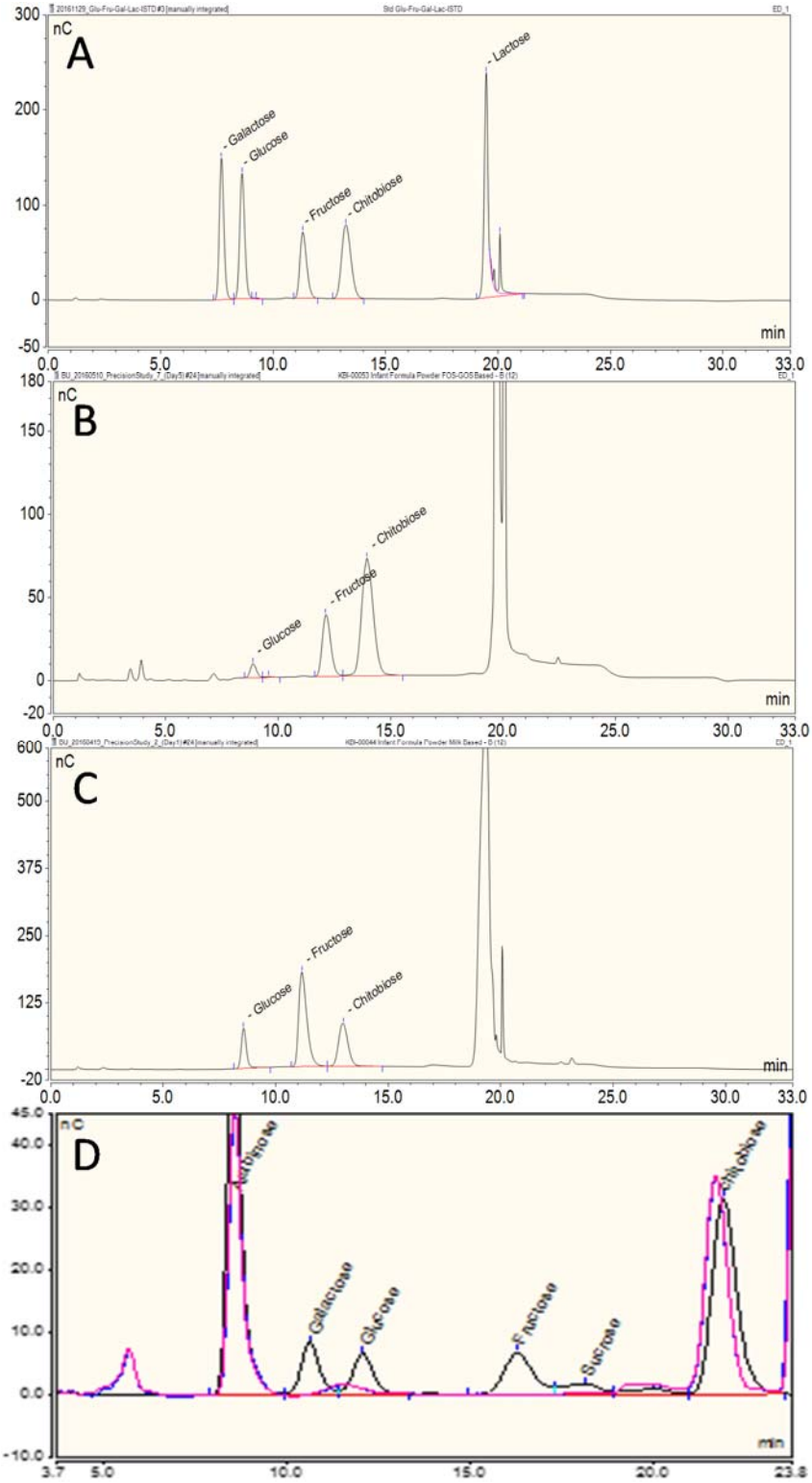

Figure 1: Example chromatograms: A: Standards separated on the PA20; B: Analysis of formula containing around 0.03 g/100g fructan on PA20; C: Analysis of formula containing around 0.28 g/100g fructan on PA20; D Standards separated on the PA1

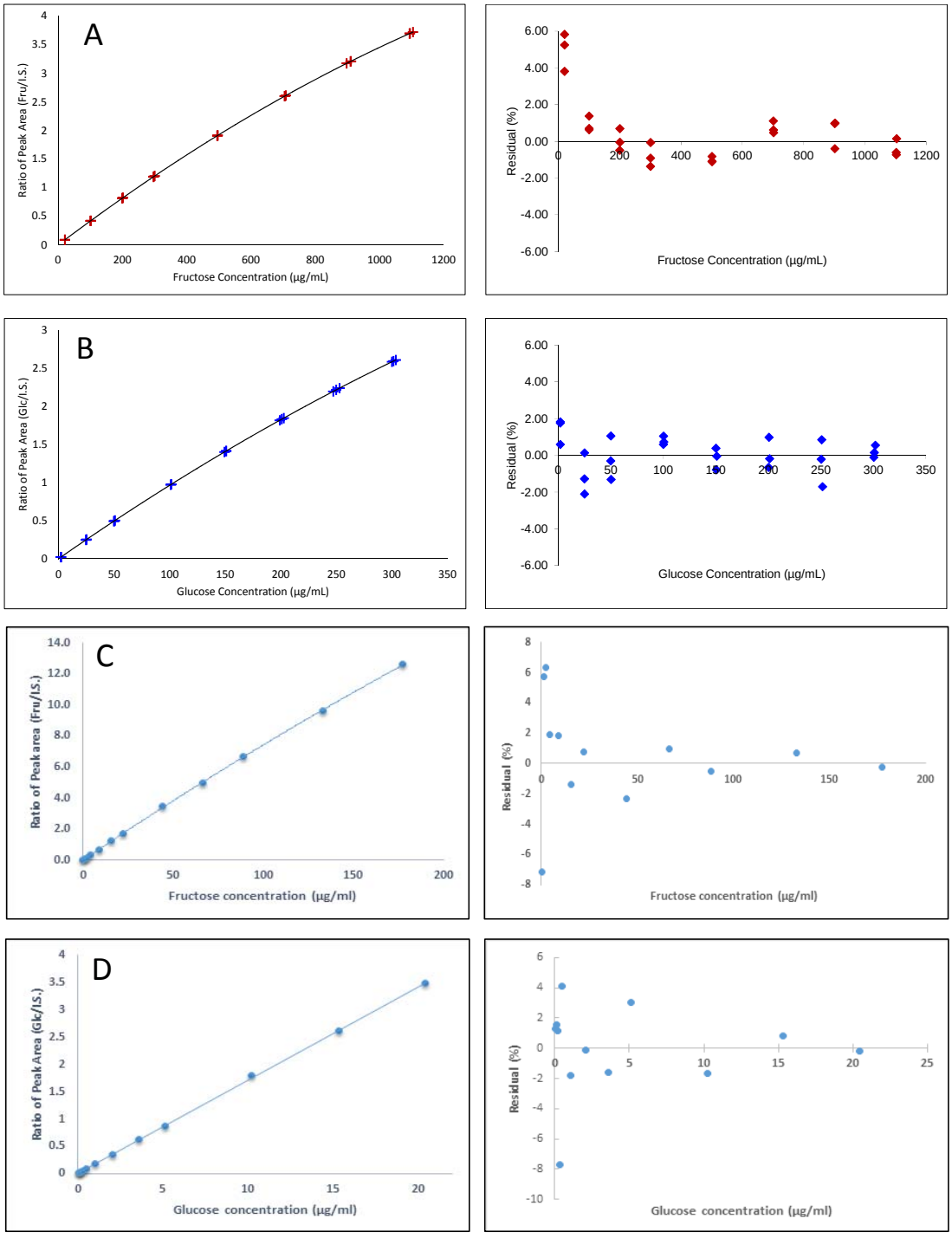

Figure 2: Calibration curves using chitobiose as internal standard (I.S.) (left) and plots of the relative residuals (right). A: Fructose at NRC; B Glucose at NRC; C Fructose at CCC; D Glucose at CCC

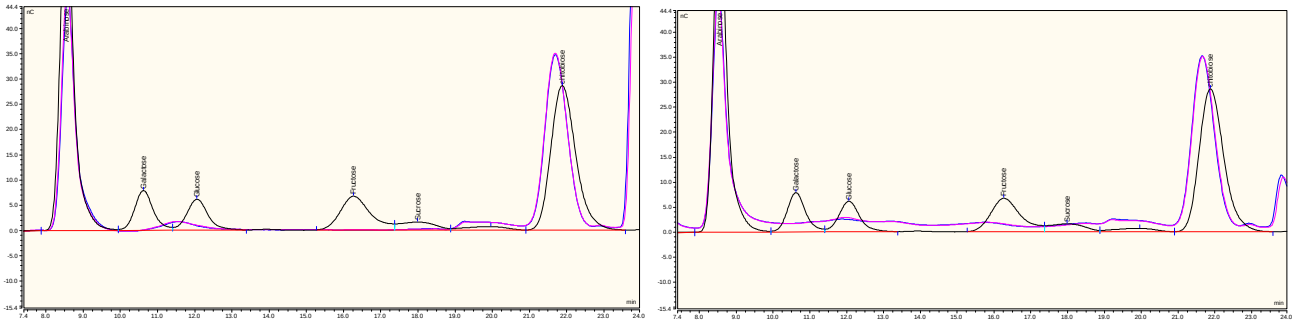

Figure 3: *Chromatograms of GOS (left) and polydextrose (right). In blue the chromatogram with no A0 correction, in pink the chromatogram with A0 correction and in black the standards arabinose, galactose, glucose, fructose, sucrose and chitobiose (internal standard)).*