FOOD COMPOSITION AND ADDITIVES

Determination of Fructan (Inulin, FOS, Levan, and Branched Fructan) in Animal Food (Animal Feed, Pet Food, and Ingredients): Single-Laboratory Validation, First Action 2018.07

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Traditional enzyme-based methods for measurement of fructan were designed to measure just inulin and branched-type (agave) fructans. The enzymes employed, namely exo-inulinase and endo-inulinase, give incompletely hydrolysis of levan. Levan hydrolysis requires a third enzyme, endo-levanase. This paper describes a method and commercial test kit (Megazyme Fructan Assay Kit) for the determination of all types of fructan (inulin, levan, and branched) in a variety of animal feeds and pet foods. The method has been validated in a single laboratory for analysis of pure inulin, agave fructan, levan, and a range of fructan containing samples. Quantification is based on complete hydrolysis of fructan to fructose and glucose by a mixture of exo-inulinase, endo-inulinase, and endo-levanase, followed by measurement of these sugars using the PAHBAH reducing sugar method which gives the same color response with fructose and glucose. Before hydrolysis of fructan, interfering sucrose and starch in the sample are specifically hydrolyzed and removed by borohydride reduction. The single-laboratory validation (SLV) outlined in this document was performed on commercially available inulin (Raftiline) and agave fructan (Frutafit®), levan purified from Timothy grass, two grass samples, a sample of legume hay, two animal feeds and two barley flours, one of which (Barley MAX®) was genetically enriched in fructan through plant breeding. Parameters examined during the validation included working range, target selectivity, recovery, LOD, LOQ, trueness (bias), precision (repeatability and intermediate precision), robustness, and stability. The method is robust, quick, and simple.

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This method was approved by the AOAC Expert Review Panel for Fructan as First Action.
The Expert Review Panel on Fructan invites method users to provide feedback on the First Action methods. Feedback from method users will help verify that the methods are fit-for-purpose and is critical for gaining global recognition and acceptance of the methods. Comments can be sent directly to the corresponding author or methodfeedback@aoac.org.
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Fructans are defined as any compound where one or more fructosyl-fructose linkage constitutes a majority of the linkages. This refers to polymeric material as well as oligomers as small as the trisaccharide kestose. Material included in this definition may or may not contain D-glucosyl substituents (1). Fructans are widely distributed in the plant kingdom. They are present in monocotyledons, dicotyledons, and in green algae. Fructans differ in molecular structure and in molecular weight. They may be classified into three of the following main types: the inulin group, the levan group, and the branched (agave-type) group (2, 3). The inulin group consists of material that has mostly or exclusively the (2 → 1) fructosyl-fructose linkage. Levan is material that contains mostly or exclusively the (2 → 6) fructosyl-fructose linkage. The branched group has both (2 → 1) and (2 → 6) fructosyl-fructose linkages in significant amounts (e.g., graminan from Gramineae and agave fructan; 2, 3).

AOAC Official Method 2018.07
Fructan (Inulin, FOS, Levan, and Branched Fructan) in Animal Food (Animal Feed, Pet Food, and Ingredients) First Action 2018

This method is based on AOAC *Official Method of Analysis* **999.03** (4–6), which is commercially available from Megazyme as the Fructan Assay Kit (Megazyme Cat. No. K-FRUC).

A. Method

(a) *Scope of the method*.—(*1*) *Target analyte*.—Fructan, including inulin, fructo-oligosaccharide (FOS; nonreducing), levan (7), and branched fructans (e.g., agave fructan).

(*2*) *Matrixes*.—For this validation, the Fructan Assay Kit (K-FRUC) was tested with the multiple matrixes.—(*a*) *Animal feed and pet food samples*.—Brett Brothers swine feed (Glanbia, Ireland), Purina GoCat dry cat food (Tesco Ireland, Greystones, Ireland), Top Spec TopChop alfalfa (TopSpec Equine Ltd, Thirsk, United Kingdom), Timothy grass hay (Lot No. 171201; Grass Seed Direct, Menstrie, Scotland), and Rye grass hay (Seed Phenomics and Quality Traits Agriculture Victoria Research, Horsham, Australia).

(*b*) *Barley grain/flour*.—Conventional barley flour (Lot No. 60301a; Megazyme, Bray, Ireland) and Barley MAX® (The Healthy Grain Pty. Ltd, South Yarra, Australia).

(*c*) *Pure or high purity commercial of purified fructans*.—Raftiline® Native Chicory Inulin (BENEO Tienen, Tienen, Belgium), Frutafit agave fructan (Sensus, Roosendaal,

The Netherlands), and Levan (Cat. No. P-LEVAN; Lot No. 171114 purified from Timothy grass; Megazyme).

(*3*) *Certified reference materials (CRMs)*.—CRMs are not currently available for fructan.

(*4*) *Expression of results*.—The concentration of fructan is expressed as percent w/w of the sample analyzed.

B. Principle

Products to be analyzed are suspended in water in tubes that are heated in a boiling water bath to dissolve the fructan. Sucrose in the sample is hydrolyzed at pH 6.5 and 30°C to D-glucose and D-fructose by a specific sucrase enzyme, which has no action on lower degrees of polymerization (DP) FOS such as 1-kestose and 1,1-kestotetraose. Starch and maltodextrins are concurrently hydrolyzed to maltose and maltotriose by pullulanase and β-amylase at pH 6.5 and 30°C, and these oligosaccharides are then hydrolyzed to D-glucose by maltase. D-Glucose and D-fructose are reduced at 40°C by sodium borohydride to the corresponding sugar alcohols, D-sorbitol and D-mannitol. Native fructans and nonreducing FOS such as Neosugars® are not affected by this reaction because they are nonreducing. Fructans and FOS are specifically hydrolyzed by exo- and endo-inulinase and endo-levanase at 40°C to D-glucose and D-fructose. D-Fructose and D-glucose derived from fructan are measured using the *p*-hydroxybenzoic acid hydrazide (PAHBAH) reducing-sugar method. This method is simple to use and the color response for D-fructose and D-glucose is the same (Figure **2018.07**).

C. Equipment

(**a**) *Grinding mill*.—Centrifugal, with 12 tooth rotor and 0.5 mm sieve, or similar device. Alternatively, cyclone mill may be used for small laboratory samples.

(**b**) *Microfuge*.—Required speed 13 000 rpm.

(**c**) *Disposable 2.0 mL polypropylene microfuge tubes*.—Sarstedt Cat. No. 72.691 (Sarstedt Ltd., Drinagh, Co. Wexford, Ireland), or equivalent.

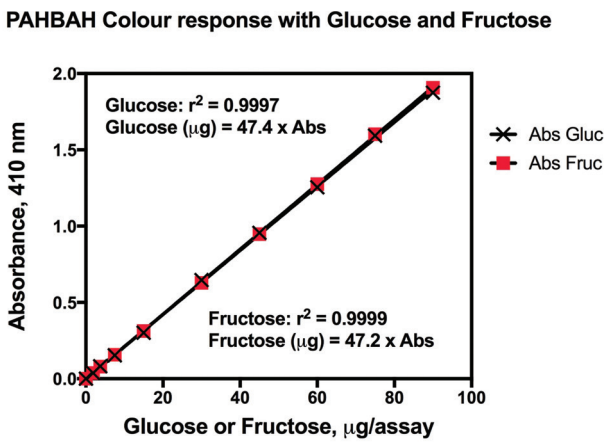

Figure 2018.07. Linearity of the PAHBAH color reaction with fructose and glucose. (x) Glucose; glucose (mg) = 47.4 × absorbance; r^2 = 0.9997. (O) Fructose; fructose (mg) = 47.2 × absorbance; r^2 = 0.9999.

(**d**) *Glass test tubes*.—16 × 120 mm, round bottom, 17 mL.

(**e**) *Pyrex screw-cap culture tubes (25 × 150 mm)*.—With PTFE-lined phenolic caps (e.g., Fisher Cat. No. 14-933D; Fisher Scientific, Dublin, Ireland).

(**f**) *Volumetric flasks*.—50 and 100 mL.

(**g**) *Micropipettors*.—Gilson Pipetman® (100 and 200 µL), Woodside Industrial Estate (Dunstable, United Kingdom), or equivalent.

(**h**) *Positive-displacement pipettor*.—Brand HanyStep® S with 5.0 mL Brand PD-Tip® (to dispense 0.2 mL aliquots sucrase mixture, 0.1 mL aliquots fructanase, and other solutions and buffers) with 50 mL Brand PD-Tip (to dispense 5.0 mL aliquots PAHBAH working reagent), Sigma-Aldrich Ireland, Ltd (Arklow, Ireland), or equivalent.

(**i**) *Bottle-top dispenser*.—Brand S Digital 2.5–25 mL Cat. No. 4600351 to dispense 25 mL of 200 mM sodium acetate buffer (pH 5.0), Sigma-Aldrich Ireland Ltd, or equivalent.

(**j**) *Water bath*.—Maintaining 30 ± 0.1°C and 40 ± 0.1°C.

(**k**) *Boiling water bath*.—Deep-fat fryer filled with water at 95–100°C, or equivalent.

(**l**) *Analytical balance*.—Accurate to 0.001 g.

(**m**) *Spectrophotometer*.—Operating at 410 nm.

(**n**) *Freeze-drier*.—Virtis Genesis® 25XL (Biopharma Process Systems, Winchester, United Kingdom), or equivalent.

(**o**) *Vortex mixer*.

(**p**) *pH meter*.

(**q**) *Stop-clock timer*.

D. Chemicals and Reagents

All reagents should be of analytical purity grade.

(**a**) *Sodium maleate buffer*.—100 mM, pH 6.5. Dissolve 11.6 g maleic acid in 900 mL distilled water and adjust pH to 6.5 with 2 M NaOH (8.0 g NaOH/100 mL) and dilute to volume in 1 L volumetric flask with water. Store at 4°C.

(**b**) *Sodium acetate buffer*.—100 mM, pH 4.5. Pipet 5.8 mL glacial acetic acid (1.05 g/mL) into 900 mL distilled water. Adjust to pH 4.5 using 1 M NaOH and dilute to 1 L with water. Store at 4°C.

(**c**) *p-Hydroxybenzoic acid hydrazide (PAHBAH) reducing-sugar assay reagent*.—(*1*) *Solution A*.—Add 10 g PAHBAH (e.g., Sigma Cat. No. H-9882; Sigma Chemical Co., St. Louis, MO) to 60 mL water in a 250 mL beaker on magnetic stirrer. Stir slurry and add 10 mL concentrated HCl. Adjust to 200 mL with distilled water and store at room temperature (approximately 22°C). Solution is stable for at least 2 years.

(*2*) *Solution B*.—Add 24.9 g trisodium citrate dihydrate to 500 mL distilled water and stir to dissolve. Add 2.20 g $CaCl_2 \cdot 2H_2O$ and dissolve by stirring. Then, add 40.0 g NaOH and dissolve with stirring. (Solution may be milky but will clarify on dilution.) Adjust volume to 2 L. The solution is stable for at least 2 years at room temperature (approximately 22°C).

(*3*) *PAHBAH working reagent*.—Immediately before use, add 20 mL Solution A to 180 mL Solution B and mix thoroughly. This solution should be stored on ice and is stable for approximately 4 h.

(**d**) *Sodium hydroxide (50 mM)*.—Dissolve 2.0 g NaOH in 900 mL distilled water. Adjust volume to 1 L. Store at room temperature (approximately 22°C).

(**e**) *Alkaline borohydride*.—Accurately weigh approximately 50 mg sodium borohydride (Sigma Cat. No. S-9125) into

polypropylene containers (10 mL volume with screw cap). Record weight on tubes (approximately 10 for convenience), seal tubes, and store in a desiccator for future use. Immediately before use, dissolve sodium borohydride (at 10 mg/mL) in 50 mM NaOH solution, **D(d)**. This solution is stable for 4–5 h at room temperature. *Note:* See safety note relating to the use of sodium borohydride.

(**f**) *Acetic acid (200 mM)*.—Add 11.5 mL glacial acetic acid to distilled water and adjust volume to 1 L. Store at room temperature (approximately 22°C).

(**g**) *Sucrase/amylase mixture (Sucrase/β-amylase/pullulanase/maltase)*.—12 U sucrase/mL. Dissolve contents of one vial containing sucrase (270 U) plus β-amylase (*Bacillus cereus*, 320 U), pullulanase (*B. licheniformis*, 64 U), and maltase (yeast, 1600 U; as a freeze-dried powder) in 22 mL sodium maleate buffer, **D(a)**. Divide enzyme solution into 5 mL aliquots and store frozen in polypropylene containers to prevent microbial contamination. If not diluted in buffer, the freeze-dried enzyme is stable 5 years when stored below –10°C. One unit sucrase activity is the amount of enzyme required to release 1 μmole glucose/min from sucrose at pH 6.5 and 30°C.

(h) *Fructanase solution*.—360 U/mL exo-inulinase, 11 U/mL endo-inulinase, and 7.3 U/mL endo-levanase. Dissolve contents of one vial containing 8000 U exo-inulinase, 240 U endo-inulinase, and 160 U endo-levanase in 22 mL sodium acetate buffer, **D(b)**. Divide enzyme solution into 5 mL aliquots and store frozen in polypropylene containers to prevent microbial contamination. If not diluted in buffer, the freeze-dried enzyme is stable 5 years when stored below –10°C. One unit *exo*-inulinase activity is the amount of enzyme required to release 1 μmole reducing-sugar equivalents (as fructose) per minute from kestose (10 mg/mL) at pH 4.5 and 30°C.

(**i**) *Inulin-control powder*.—Containing a known amount of fructan (inulin) from dahlia tubers freeze-dried in the presence of α-cellulose, and it is stable when stored dry at room temperature.

(**j**) *Levan control powder*.—Containing a known amount of levan from Timothy grass freeze-dried in the presence of α-cellulose, and it is stable when stored dry at room temperature.

(**k**) *Sucrose control powder*.—Sucrose freeze-dried in the presence of α-cellulose, and it is stable when stored dry at room temperature (approximately 22°C).

(**l**) *D-Fructose standard stock solution*.—1.5 mg/mL in 0.2% benzoic acid solution. Before preparing solution, dry powdered crystalline fructose (purity >97%) 16 h at 60°C under vacuum.

Items **D(g–l)** are supplied in the K-FRUC available from Megazyme, but preparations of enzymes and standards which meet these criteria may also be used.

E. Safety Considerations

The general safety measures that apply to all chemical substances should be adhered to. For more information regarding the safe use and handling of the Fructan Assay Kit, please refer to the associated sodium dodecyl sulfate (SDS) that is available from the Megazyme website, http://www.megazyme.com. Sodium borohydride releases hydrogen on contact with water and is extremely hazardous in terms of skin or eye contact. The analyst should wear safety gloves and glasses when weighing this chemical and refer to the appropriate SDS.

F. Preparation of Test Materials

All products should be equilibrated to room temperature (22°C) before they are weighed.

(**a**) *Test samples*.—For dry food, animal feed or grass samples, grind approximately 50 g sample in a grinding mill to pass 0.5 mm sieve. Transfer all material into wide-mouthed plastic jars and mix well by shaking and inversion. For wet products such as canned pet foods, homogenize samples with a Waring blender and freeze-dry approximately 100 g. Analyze a representative sample of the dry product. Record sample at both wet weight and dry weight.

(**b**) *Fructose standard working solution*.—Add 0.2 mL fructose standard stock solution [1.5 mg/mL, **D(l)**] to 0.9 mL acetate buffer, **D(b)**, and mix thoroughly. Dispense 0.2 mL aliquots solution (containing 54.5 μg fructose) in quadruplicate to the bottoms of four glass test tubes, **C(d)**. Add 0.1 mL acetate buffer, **D(b)**, to each tube. Immediately before incubation in boiling water bath, add 5.0 mL PAHBAH working reagent, **D(c)***(3)*.

(**c**) *Reagent blank*.—Transfer 0.3 mL acetate buffer, **D(b)**, into test tubes and proceed with the standard assay procedure, **G(c)***(3)*.

(**d**) *Sucrose control powder [containing approximately 10% (w/w) sucrose]*.— Extract and analyze 400 mg powder per **G(a)***(1)*. This product contains no fructan and is used to check the effectiveness of the sucrase and borohydride treatments. The calculated fructan content should be less than 0.3% (w/w).

(**e**) *Inulin and levan control powders*.—Extract and analyze 100 mg control powders per **G(a)***(2)*.

G. Assay Procedure

(a) *Extraction of fructan*.—(*1*) *Products containing 0–10% (w/w) fructan*.—Run D-fructose working standard solution (in quadruplicate), reagent blank (in duplicate), fructan control flour, and sucrose control flour with each set of tests. Use a reagent blank to zero the spectrophotometer.

(*a*) Accurately weigh approximately 400 mg test portion of sample into a dry pyrex screw-cap culture tube (25 × 150 mm) and add 25 mL distilled water. Loosely cap the tube. Place the tube into a boiling water bath and heat for a total of 10 min. After 5 min, tighten the tube cap and vigorously mix the contents on a vortex mixer. Return the tube to the boiling water bath. After another 5 min, remove the tube from the boiling water and mix the contents by inversion and shaking.

(*b*) Cool the solution to room temperature and mix thoroughly. Transfer 2.0 mL into a disposable 2.0 mL polypropylene microfuge tube, **C**(**c**).

(*c*) Centrifuge the solution at 13 000 rpm for 5 min in a microfuge, **C**(**b**), and analyze within 1 h after centrifugation. The supernatant may be slightly turbid. This is not a problem. (If the solution is stored for several hours at low temperature before analysis, the fructan may precipitate from solution. In such cases, reheat solution to approximately 80°C and let cool to room temperature before removing solutions for analysis.) Use this solution for determination of fructan per **G(b)***(1)*.

(*2*) *Products containing 10–40% (w/w) fructan*.—Run D-fructose working standard solution (in quadruplicate), reagent blank (in duplicate), fructan control flour, and sucrose control flour with each set of tests. Use a reagent blank to zero the spectrophotometer.

(a) Accurately weigh approximately 100 mg sample into a dry pyrex screw-cap culture tube (25 × 150 mm) and add 25 mL distilled water. Loosely cap the tube. Place the tube into a boiling water bath and heat for a total of 10 min. After 5 min, tighten the tube cap and vigorously mix the contents on a vortex mixer. Return the tube to the boiling water bath. After an additional 5 min, remove the tube from the boiling water bath and mix the contents by inversion and shaking.

(*b*) Cool solution to room temperature and mix thoroughly. Transfer 2.0 mL into a disposable 2.0 mL polypropylene microfuge tube, **C**(**c**).

(*c*) Centrifuge the solution at 13 000 rpm for 5 min in a microfuge, **C**(**b**), and analyze within 1 h after centrifugation. The supernatant may be slightly turbid; this is not a problem. (If the solution is stored for several hours at low temperature before analysis, the fructan may precipitate from solution. In such cases, reheat solution to approximately 80°C and let cool to room temperature before removing solutions for analysis.) Use this solution for determination of fructan per **G(b)***(1)*.

(**b**) *Removal of sucrose, starch, and reducing sugars*.—(*1*) Accurately transfer a 0.2 mL aliquot filtrate to be analyzed (containing approximately 0.1–1.0 mg/mL fructan, or controls) to bottom of a glass test tube, **C(d)**.

(*2*) Add 0.2 mL diluted sucrase/amylase solution, **D(g)**, to each tube and incubate at 30°C for 30 min.

(*3*) Add 0.2 mL alkaline borohydride solution, **D(e)**, to each tube. Stir the tube vigorously, cover them with Parafilm®, and incubate at 40°C for 30 min for complete reduction of reducing sugars to sugar alcohols.

(*4*) Add 0.5 mL acetic acid, **D(f)**, to each tube with vigorous stirring on a vortex mixer. If borohydride solution is saturating, a vigorous effervescence should be observed. If not, there is a problem with the borohydride. If this is the case, repeat the analysis with freshly prepared borohydride solution. (This treatment removes excess borohydride and adjusts pH to approximately 4.5.) This is Solution S.

(**c**) *Hydrolysis and measurement of fructan*.—(*1*) Transfer 0.2 mL aliquots Solution S (in triplicate) to the bottoms of glass test tubes, **C(d)**.

(*2*) Add 0.1 mL fructanase solution, **D(h)**, to two of these tubes, stir contents on vortex mixer, and incubate at 40°C for 30 min for complete hydrolysis of fructan to fructose and glucose. To the third tube (the sample blank), add 0.1 mL of 100 mM sodium acetate buffer, **D(b)**.

(*3*) Add 5.0 mL PAHBAH working reagent to all tubes, including the fructose standard working solution, **F(b)**; reagent blank, **F(c)**; extracts of fructan control powder, **F(e)**; and sucrose control powder, **F(d)**, and incubate in boiling water bath for exactly 6 min.

(*4*) Remove all tubes from the boiling water bath and immediately place in cold water (18–20°C) for approximately 5 min.

(*5*) Measure the absorbances of all solutions at 410 nm against reagent blank as soon as possible after cooling. The PAHBAH color complex will fade with time. At room temperature, little change (<5%) is seen over 10–15 min. The same change will be seen in the standard solutions.

H. Calculations

Calculate total fructan content (percent w/w, on "as-is" basis) in test samples as follows:

$$\text{Total fructan, \% (w/w "as is")} = \Delta A \times F \times 5 \times 25 \times \frac{1.1}{0.2} \times \frac{100}{W} \times \frac{1}{1000} \times \frac{162}{180} \times D$$

$$= \Delta A \times \frac{F}{W} \times D \times 61.9$$

where ΔA = absorbance of 0.2 mL reaction sample solutions – the absorbance for the sample blank read against reagent blank; F = factor to convert absorbance values to micrograms fructose (i.e., 54.5 μg fructose/absorbance value for 54.5 μg fructose); 5 = factor to convert from 0.2 mL as assayed to 1.0 mL; 25 = volume (milliliters) of extractant used; 1.1/0.2 = 0.2 mL was taken from 1.1 mL enzyme digest for analysis; W = weight (milligrams) of test portion extracted; 100/W = factor to express fructan as percentage of flour weight; 1/1000 = factor to convert from micrograms to milligrams; 162/180 = factor to convert from free fructose, as determined, to anhydrofructose (and anhydroglucose), as occurs in fructan; and D = further dilution of the sample extract.

Note: In each FOS molecule, the terminal sugar is not anhydro (i.e., has a molecular weight of 180). In the calculations, the factor 162/180 applies only to anhydro-sugars. However, because the average DP of FOS in most plant roducts (e.g., chicory, Timothy grass, and agave) is >10, then the underestimation of FOS is, at most, [(162 × 9 + 180) – (162 × 10)] / [162 × 10] × 100 = 1.1% of the fructan content.

Note: These calculations can be simplified by using the Megazyme Mega-Calc™ Excel-based calculator downloadable from the product page where this appears on the Megazyme website (http://www.megazyme.com).

I. Indicative Controls

Indicative controls are used as a check on assay conditions. The final absorbance of the sucrose/α-cellulose control powder should be very low (<0.08), which demonstrates the effectiveness of both the sucrase treatment step and the borohydride reduction step. If sucrose is not completely hydrolyzed by the sucrase/amylase treatment, it will then be hydrolyzed by the fructanase mixture and give erroneously high fructan values. Other indicative controls can be used, such as soluble starch, β-glucan, and α-cellulose. The absorbance from α-cellulose should be negligible, i.e., <0.02; for β-glucan, and for soluble starch, the absorbance should be very low, i.e., <0.05. This result for starch demonstrates the effectiveness of the borohydride reduction step.

Validation

Planning

The purpose of this report is to verify and validate a recently updated fructan assay procedure developed and supplied by Megazyme as detailed in the K-FRUC booklet (K-FRUC). This method is an extension of AOAC Method **999.03** (measurement of total fructans in foods using the enzymatic/spectrophotometric method). endo-Levanase has been added to the fructanase mixture (which also contains exo-inulinase and endo-inulinase). The presence of endo-levanase facilitates quantitative measurement of levan as well as inulin and branched fructans.

Performance Characteristics

Performance characteristics that are investigated within this study are working range, target selectivity (inulin, levan, branched fructans, and FOS), specificity (removal of interfering sugars including sucrose; galactosyl-sucrose oligosaccharides, e.g., raffinose and stachyose; starch; maltodextrins; and reducing sugars), LOD, LOQ, trueness (bias) and precision (repeatability and intermediate precision).

Working range.—The assay follows the Megazyme K-FRUC and has a working range of 0.1 to 100% w/w fructan in the sample. For samples containing 0–10% (w/w) fructan, 400 mg sample is extracted with 25 mL hot water to give a fructan content of 0.1–1.6 mg/mL; equivalent to approximately 3.6–58.2 μg sugar in the PAHBAH assay mixture. For samples containing 10–40% (w/w) fructan, 100 mg sample is extracted with 25 mL hot water to give fructan concentrations of 0.4–1.6 mg/mL, which is equivalent to approximately 14.4–58.2 μg sugar in the PAHBAH assay mixture. For samples containing 40–100% (w/w) fructan content, 100 mg sample is extracted with 25 mL hot water, and on cooling, an aliquot (1 mL) this extract is diluted by adding to 2 mL water (3-fold dilution), to give fructan concentrations of 0.53–1.3 mg/mL; equivalent to approximately 19.3–47.3 μg sugar in the PAHBAH assay mixture.

The linearity of the PAHBAH color reaction with fructose and glucose is shown in Figure **2018.07**. The linear range for fructose and glucose reached 90 μg sugar in the PAHBAH assay mixture with r^2 values of 0.9997 and 0.9999, respectively. Also, the color response with the two sugars is identical. The linearity of pure fructan samples is shown in Figure 1.

Selectivity.—The fructan assay procedure is specific for fructan (inulin, FOS, branched fructans, and levan) if performed as described. Under incubation conditions similar to those employed in the fructan assay procedure (same enzyme concentration, but with fructan concentration approximately 40-fold higher than used in the assay) the mixture of endo-inulinase, endo-levanase, and exo-inulinase

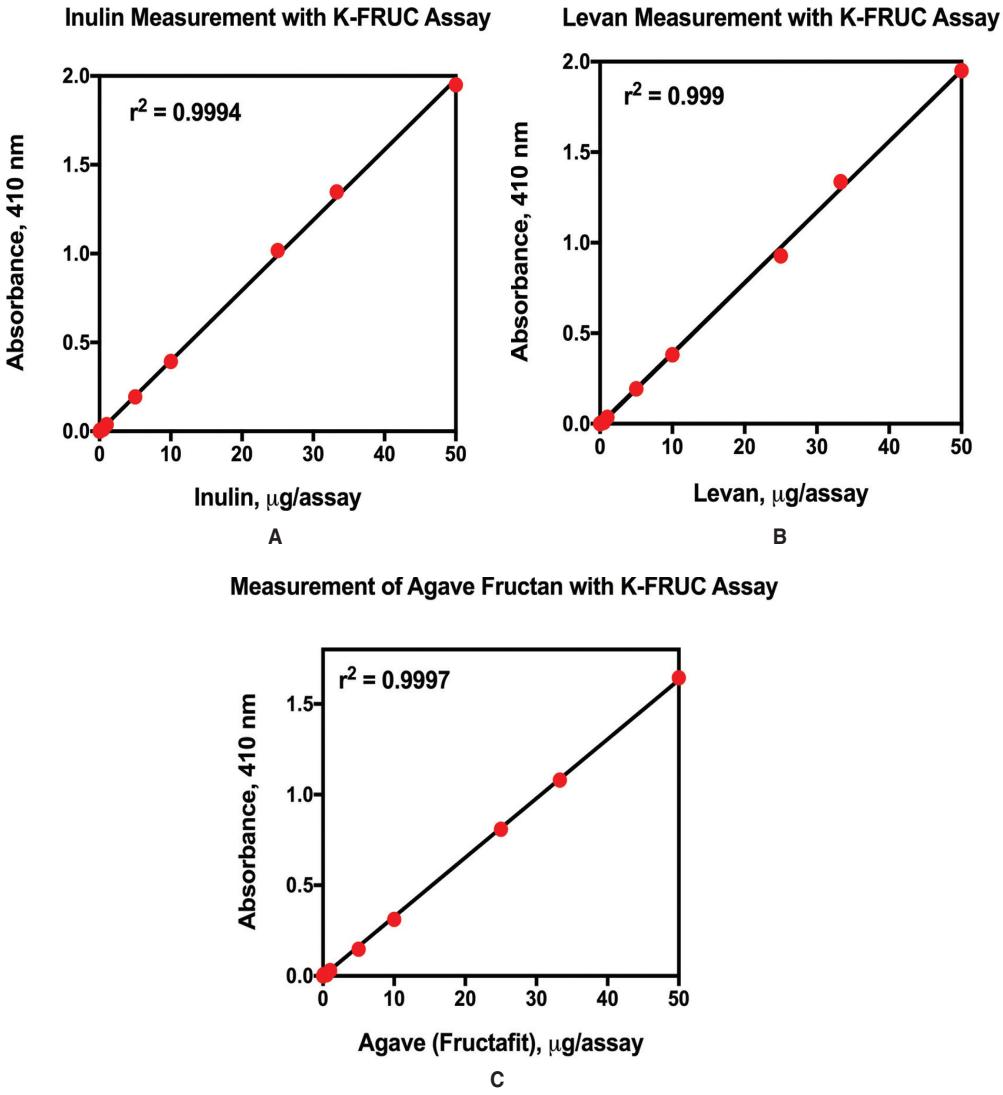

Figure 1. Linearity of inulin, levan, and agave fructan determination using the K-FRUC assay procedure. The inulin and levan used were 98% pure (w/w; dry-weight basis), whereas agave fructan was 82% pure (w/w; dry-weight basis). (A) Chicory inulin, r^2 = 0.9994; (B) Levan from Timothy grass, r^2 = 0.999; and (C) agave fructan (Agavafit®), r^2 = 0.9997.

used gave complete hydrolysis of inulin, FOS, levan, and branched fructan (agave) to fructose and glucose (Figure 2). Samples of Raftilose®, Neosugars, agave fructan, and Timothy levan were analyzed by HPLC by gel permeation chromatography using TSKgel® G2500PW$_{XL}$ columns both before and after incubation with the fructanase mixture as used in the fructan assay procedure. Clearly, each of the fructans was completely hydrolyzed to fructose and glucose within 20 min.

Specificity.—In the analysis of fructans in food or feed samples, sucrose will also be measured if it is first not removed. Sucrose is removed in the prehydrolysis step with the sucrase/amylase mixture by hydrolysis to fructose and glucose. In this sucrase/amylase incubation step, starch is also hydrolyzed (to glucose) and subsequently removed by borohydride reduction. Starch is removed, not because it is hydrolyzed by the fructanase mixture used in the assay, but because it is partially hydrolyzed in the PAHBAH incubation step (because of the highly alkaline nature of the reagent), which would give erroneously higher fructan values. Galactosyl-sucrose oligosaccharides (raffinose, stachyose, and verbascose as found in legume seeds) are measured as fructan in the fructan assay procedure if they are not removed. Removal of these oligosaccharides is achieved by incubating the sample extract with α-galactosidase at pH 4.5 before incubation with the sucrase/amylase mixture. In this step, the galactosyl-sucrose oligosaccharides are hydrolyzed to galactose and sucrose by α-galactosidase, with subsequent hydrolysis of sucrose to glucose and fructose by sucrase. Interference by sucrose, wheat starch, maltodextrins, α-cellulose, β-glucan, and galactosyl-sucrose oligosaccharides is shown in Table 1. Each of the samples was analyzed using the full fructan assay procedure. Clearly, starch, maltodextrins, α-cellulose, and β-glucan do not interfere with the assay.

Recovery.—For accurate measurement of fructan and FOS, the sucrase/amylase mixture must give near complete hydrolysis of sucrose with essentially no hydrolysis of fructan or FOS. The recovery of kestose, the smallest and most sensitive native FOS, in the fructan assay procedure is shown in Table 2. Assays were performed according to the standard procedure, and also with the noninclusion of the sucrase/amylase incubation step.

Under the incubation conditions used, sucrose is essentially completely hydrolyzed and removed and there is negligible hydrolysis of kestose.

In order to determine the effect of matrix on recovery of fructan, a variety of food and feed samples were spiked with inulin (and in one case, levan; Tables 3 and 4). A range of animal food and feed samples were spiked with three levels of inulin and analyzed to obtain recovery values. GoCat dry cat food and Timothy grass were spiked with known quantities of either inulin or levan and recoveries were determined by analyzing and comparing fructan values for the sample, with and without the added spike. Timothy grass samples with two levels of

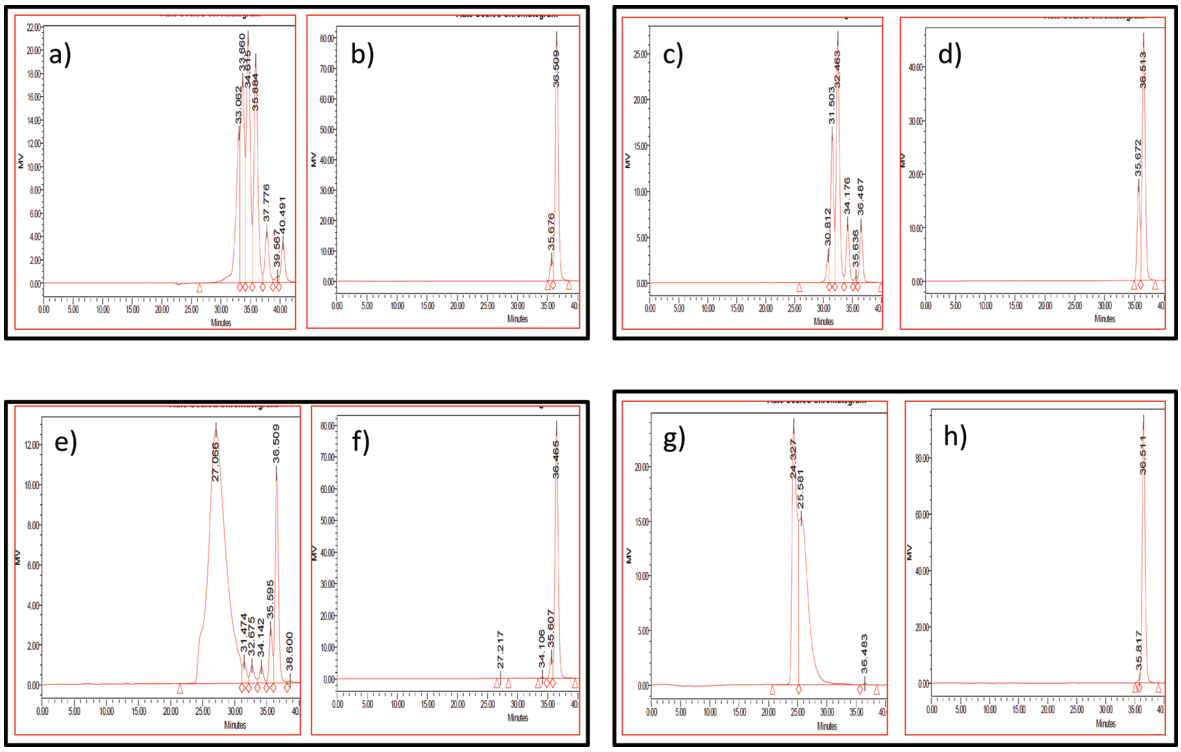

Figure 2. Hydrolysis of various fructans by the fructanase mixture used in the Megazyme Fructan Assay Kit (Cat. No. K-FRUC). Aliquots of fructan solution (1 mL, 10 mg/mL) in 20 mM sodium acetate buffer (pH 4.5) were incubated with 1.0 mL fructanase solution from K-FRUC kit at 40°C. Reactions were terminated after 20 min by heating the solution at 100°C for 5 min. Reaction blanks were prepared by heating the enzyme solution at 100°C for 5 min before adding the fructan solution. Solutions were centrifuged at 13 000 rpm for 5 min and the supernatant solution analyzed by chromatography on TSKgel G2500PWXL HPLC columns with in-line deionization. (a) Raftilose at 0 min; (b) Raftilose at 20 min; (c) Neosugars at 0 min; (d) Neosugars at 20 min; (e) agave fructan at 0 min; (f) agave fructan at 20 min; (g) Timothy levan at 0 min; and (h) Timothy levan at 20 min.

Table 1. Results of the SLV for the Fructan Assay Kit

	SMPR 2018.002	K-FRUC
Operating range, % (w/w)	0.2–100	0.21–98.4[a]
LOQ, % (w/w)	0.20	0.119[b]
RSD_r, % (0.2–1%, w/w fructan)	7	4.74
RSD_r, % (>1–10%, w/w fructan)	5	3.59
RSD_r, % (>10–100%, w/w fructan)	3	2.96
RSD_R, % (0.2–1%, w/w fructan)	14	8.47
RSD_R, % (>1–10%, w/w fructan)	10	6.36
RSD_R, % (>10–100%, w/w fructan)	6	5.77

[a] Precise range dictated by fructan content in samples tested.

[b] Based on replicate measurements for a sample with approximately 1% (w/w) fructan.

Table 2. Interference of α- and β-glucans, maltodextrins, and galactosyl-sucrose oligosaccharides on fructan determination using the Fructan Assay Kit

Sample	Sample weight analyzed, mg	Apparent "fructan" content, % (w/w; "as is")
Sucrose/cellulose control (8.9% sucrose)	400	0.13 – 0.14
Wheat starch	400	0.03 – 0.05
Maltodextrins (Matsutani Chemical Co.)	400	0.08 – 0.10
β-Glucan (Megazyme Cat. No. P-BGBM)	200	0.05 – 0.06
α-Cellulose	400	0.01 – 0.02
Raffinose (galactosyl-sucrose)	7.5	7.5 – 7.7
Raffinose pretreated with α-galactosidase[a]	7.5	0.11 – 0.13
Mung beans (containing 3.05% galactosyl-sucrose oligosaccharides)	400	2.85 – 3.05
Mung beans (3.05% galactosyl-sucrose oligosaccharides) plus α-galactosidase[a]	400	0.10 – 0.11

[a] Sample (0.2 mL) was incubated with 0.05 mL of α-galactosidase solution (20 U) in 50 mM sodium acetate buffer (pH 4.5) for 30 min at 40°C.

Table 3. Recovery of kestose in the Fructan Assay Kit assay procedure performed with and without the sucrase/amylase incubation step

	Rec. kestose, mg/assay		
Kestose, mg/assay	Without incubation with sucrase mixture	With incubation with sucrase mixture (according to K-FRUC)	Rec., % (w/w)
2	2.1	2.1	100.0
5	5.0	4.9	98.4
10	10.1	10.1	100.0
20	19.5	19.7	100.8
50	50.7	48.2	96.2
Sucrose	8.8	0.16	—

spike of both inulin and levan and GoCat dry cat food with a single level of spike with either inulin or levan were analyzed in duplicate by one analyst on a single occasion. The data in Tables 3 and 4 shows that obtained recoveries were within the range required by AOAC for this method.

LOD and LOQ.—LOD (the lowest level at which detection of the analyte becomes problematic) and LOQ (the lowest level at which the performance of the assay is acceptably repeatable) of the fructan assay method were calculated by performing replicate assays ($n = 16$) on a Purina GoCat dry cat food sample with a relatively low content of fructan (0.98%, w/w). The LOD was calculated to be 0.036% (w/w) using $LOD = 3 \times s'_0$, where s'_0 = the SD of replicate measurements. the LOQ was calculated as 0.119% (w/w) using $LOQ = k_Q \times s'_0$, where s'_0 = the SD of replicate measurements; and, according to The International Union of Pure and Applied Chemistry, the default value for $k_Q = 10$. The LOQ of 0.119% (w/w) meets the LOQ requirement of the AOAC *Standard Method Performance Requirements* (SMPR®) 2018.002.

Trueness (bias).—Accuracy of the K-FRUC method was assessed by comparison of the mean fructan content obtained for a suitable reference material with a specific reference value. There are no official CRMs for fructan however the reference material used for this analysis was Raftiline (native chicory inulin), which is a commercially available, almost pure, inulin. Relative bias is calculated as (*see* Table 5):

$$b(\%) = \frac{\overline{x} - \overline{x}_{ref}}{\overline{x}_{ref}} \times 100$$

where $b(\%)$ = relative bias; $\overline{x}$ = mean fructan content; and $\overline{x}_{ref}$ = specific reference value.

The accuracy of the fructan assay procedure is extremely high, with a calculated relative bias of –2.85% for a pure inulin sample.

Precision.—The precision of the fructan assay method was assessed using 10 milled samples. For each sample, duplicate extractions were processed and analyzed on four separate occasions by two independent analysts. The fructan content of the samples tested covered a working range of 0.2–98.4% (w/w) fructan. The repeatability RSD (RSD_r) and intermediate precision RSD (RSD_I) across this sample set were well within the requirements of the SMPR. The RSD_r was ≤2.96% for samples containing 10 to 100% (w/w) fructan, ≤3.59% for samples containing 1–10% (w/w) fructan, and ≤4.74% for samples containing 0.2–1% (w/w) fructan precision (Table 6).

The RSD_I was ≤5.77% for samples containing 10–100% (w/w) fructan, ≤6.36% for samples containing 1–10% (w/w) fructan, and ≤8.47% for samples containing 0.2 to 1% (w/w) fructan precision (Table 7).

The levels of repeatability and intermediate precision obtained with the fructan assay method indicate a very high level of precision and meet the requirements of the SMPR 2018.002 for all of the specified ranges of fructan.

Stability.—The K-FRUC as formulated by Megazyme comes with a 2 year stability guarantee. If the kit components are prepared and stored as described in the kit booklet, they also are stable for at least 2 years. Individual components may have longer stability guarantees; this information is available on the component label as the expiry date. Regular quality control testing is performed. As the K-FRUC has been commercially available for a number of years (excluding the endo-levanase

Table 4. Recovery of fructan (inulin) spike added to a range of food and feed samples

Sample	Fructan content of sample, % (w/w)	Added fructan, % (w/w)[a]	Rec., %	AOAC acceptable range, %
Dairy feed complete[b]	1.07	0.93	103.7	90-110
	1.07	6.95	101.6	93-107
	1.07	36.9	99.8	95-105
Hen pellets[c]	1.31	0.93	104.3	90-110
	1.31	6.95	104.2	93-107
	1.31	36.9	99.9	95-105
Low starch horse feed[d]	0.87	0.93	101.1	90-110
	0.87	6.95	95.7	93-107
	0.87	36.9	99.5	95-105
Wet dog food[e]	0.11	0.93	95.3	90-110
	0.11	6.95	101.4	93-107
	0.11	36.9	101.2	95-105
Fish Food Aquarium[f]	0.38	0.93	104.0	90-110
	0.38	6.95	98.9	93-107
	0.38	36.9	102.0	95-105
Purina Winalot dog treats[g]	1.40	0.93	103.2	90-110
	1.40	6.95	101.6	93-107
	1.40	36.9	99.8	95-105
Red onion freeze dried[h]	19.7	0.93	108.3	90-110
	19.7	6.95	96.0	93-107
	19.7	36.9	104.3	95-105
Cauliflower freeze dried[i]	0.08	0.93	99.5	90-110
	0.08	6.95	98.6	93-107
	0.08	36.9	101.3	95-105

[a] For samples where added fructan was less than 10% of the weight of the sample, a sample weight of 400 mg was analyzed. Where the added fructan was greater than 10% of the sample, a sample weight of 100 mg was analyzed.

[b] Gain Drive Dairy feed complete.

[c] Connolly's Red Mills Layee hen pellets.

[d] Top spec Ulsakind low starch horse feed.

[e] Gain premium cuts wet dog food (freeze dried).

[f] Fish Food Aquarian Gold Fish Flake Food.

[g] Purina Winalot Shapes Dog Treats (pet treats).

[h] Commercial red onions (freeze dried from Tesco supermarket).

[i] Commercial cauliflower (freeze dried from Tesco supermarket).

Table 5. Recovery of inulin (Raftiline) and levan (Timothy grass) spiked into GoCat dry cat food and into Timothy grass

Sample	Rec. of spike, % (mg added)		Rec. parameter for AOAC, %
	Inulin	Levan	
GoCat dry cat food	99.0 (27)	99.6 (26)	95–105
	97.8 (27)	95.2 (26)	95–105
Timothy grass	95.0 (22)	102.8 (21)	95–105
	99.4 (22)	97.2 (21)	95–105
Timothy grass	99.4 (11)	101.5 (11)	95–105
	103.4 (11)	95.1 (11)	95–105

enzyme, which is a new addition), the historical data available shows that the stability of each component does not vary from batch to batch (data not shown).

Robustness.—The effect of the temperature of incubation with the sucrase/β-amylase/pullulanase mixture, the fructanase

Table 6. Relative bias

Parameter	Value
Ref. value, % (w/w; fructan)	100
Mean, % (w/w; fructan)	97.15
Relative bias; *b*(%)	–2.85
n	22

Table 7. Repeatability—for each sample, duplicate extractions were processed and applied to the assay on four separate occasions by a single analyst

Sample	Fructan[a], % (w/w; CV)				Interday[a], % (RSD_r)[b]
	Day 1	Day 2	Day 3	Day 4	
Raftaline inulin (native chicory fructan)	99.8 ± 4.1	98.2 ± 1.2	98.3 ± 0	97.2 ± 1.5	98.4 ± 2.6
	2.04	0.61	0.00	0.77	1.34
P-LEVAN (Cat. No. #171114)	97.5 ± 1.6	98.3 ± 2	99.2 ± 1	98.6 ± 0.9	98.4 ± 1.7
	0.83	1.01	0.50	0.45	0.88
Frutafit agave fructan	85.7 ± 3.5	86.4 ± 2	86.8 ± 3	83.2 ± 1.5	85.6 ± 3.6
	2.07	1.17	1.75	0.91	2.10
Timothy grass (Cat. No. #171201)	14.4 ± 0.8	14.3 ± 0.1	14.8 ± 0.4	14.8 ± 0.5	14.6 ± 0.6
	2.93	0.50	1.50	1.61	2.21
Barley MAX	14.4 ± 0.2	14 ± 1.4	14.7 ± 0.7	14.3 ± 0.6	14.4 ± 0.8
	0.60	4.92	2.46	2.27	2.95
Rye grass	11 ± 0.4	10.8 ± 0.3	10.4 ± 0.6	10.4 ± 0.1	10.7 ± 0.6
	1.73	1.25	2.98	0.46	2.96
Brett Brother swine feed	2.01 ± 0.03	1.96 ± 0.01	1.98 ± 0.12	1.94 ± 0.1	1.97 ± 0.09
	0.78	0.31	3.05	2.64	2.16
Barley flour (Cat. No. #60301a)	1.7 ± 0	1.7 ± 0.1	1.7 ± 0.1	1.5 ± 0.1	1.6 ± 0.1
	0.32	2.80	3.04	2.78	3.59
Purina GoCat dry cat food	0.98 ± 0.03	0.99 ± 0.18	1.03 ± 0	0.97 ± 0.02	0.99 ± 0.09
	1.73	9.18	0.16	0.78	4.29
TopSpec alfalfa	0.2 ± 0.02	0.2 ± 0.02	0.21 ± 0.01	0.2 ± 0.03	0.2 ± 0.02
	4.20	3.82	2.91	8.53	4.74

[a] Mean ± 2 SD.

[b] The RSD_r of the Fructan Assay method was assessed using 10 milled samples. For each sample, duplicate extractions were processed and applied to the assay on each day across 4 separate days. The fructan content of the samples tested covered a working range of 0.2 to 98.4% (w/w) fructan. The RSD_r across this sample data set was extremely high, ≤2.96% for samples containing 10–100% (w/w) fructan, ≤3.59% for samples containing 1–10% (w/w) fructan, and ≤4.74% for samples containing 0.2–1% (w/w) fructan precision. This level of repeatability and precision indicates that the fructan assay method is reliable and repeatable, and therefore suited to the application of measuring total fructan in food and feed samples.

mixture and sodium borohydride were evaluated by performing incubations at both 30 and 40°C. With the sucrase/β-amylase/pullulanase mixture and the fructanase mixture, very similar results were obtained at both 30 and 40°C. The borohydride reduction is most effective if performed at 40°C. Ultimately, 30°C was chosen for incubation with the sucrase/β-amylase/pullulanase mixture because the sucrase enzyme is less stable at 40°C than at 30°C. Borohydride reduction is best performed at 40°C, as is fructanase incubation.

Discussion

The single-laboratory validation (SLV) as outlined in this report shows that the fructan assay method is fit for purpose and applicable for the measurement of fructan (inulin, FOS, levan and, branched agave fructan) either as pure ingredients or as present in a wide range of food, feed, and plant materials as required by the SMPR 2018.002.

The fructan test kit is user-friendly, and 20 samples can be analyzed by a single operator within 4 h. This SLV included investigation into a variety of performance characteristics including working range, selectivity, recovery, LOD, LOQ, trueness (bias), precision (intermediate precision and repeatability), robustness, and stability.

The fructan assay was shown to be linear over a range of 0.1–54 μg/test. For animal feed and food samples treated per the method outlined in this report, the working range of 0.1–54 μg of fructan (as fructose plus glucose) per test equates to a concentration range of 0.1–100 g/100 g of the sample analyzed. The method as described in the Fructan Assay Kit is specific for fructan (inulin, FOS, levan, and agave).

The recovery of fructan (inulin), over fructan concentrations 0.2–100 g/100 g, and from a wide range of animal feeds and pet foods, is quantitative (97–103%) over fructan concentrations 0.1–100 g/100 g. The LOD and LOQ were determined to be 0.036 g/100 g and 0.119 g/100 g, respectively. Because there are no official CRMs for fructan, the reference material used for this analysis was Raftiline (native chicory inulin), which is a commercially available, almost pure, inulin. Accuracy of the fructan method was assessed by comparison of the mean fructan content obtained for Raftaline (a suitable reference material with a specific reference value). With a calculated relative bias of –2.85% for a pure inulin sample, it is clear that the accuracy of the Fructan Assay Kit method is extremely high. Also, excellent recoveries of fructan spike were achieved for all animal feed and pet food samples tested (97–103%), and good recoveries were achieved for a high fructan vegetable sample (red onions).

Robustness testing included the examination of incubation temperature (30 and 40°C). Variations in this parameter had no influence on the results. All kit components were shown to have at least 2 years stability when stored as recommended, and both enzyme components (sucrase/β-amylase/pullulanase and

Table 8. Reproducibility—for each sample, duplicate extractions were processed and applied to the assay on four separate occasions by two independent analysts

	Fructan[a], % (w/w; CV)								
	Laboratory 1[b]				Laboratory 2[c]				
Sample	1	2	3	4	1	2	3	4	Interlab[a], % (RSD_R)
Raftaline inulin (native chicory fructan)	99.8 ± 4.1	98.2 ± 1.2	98.3 ± 0	97.2 ± 1.5	98.9 ± 0.4	104.8 ± 19.5	103.3 ± 2	101.9 ± 1.7	100.3 ± 7.4
	2.04	0.61	0.00	0.77	0.19	9.32	0.98	0.82	3.70
P-LEVAN (Cat. No. #171114)	97.5 ± 1.6	98.3 ± 2	99.2 ± 1	98.6 ± 0.9	97 ± 0.2	97.9 ± 2.3	99.8 ± 0.1	101.3 ± 1	98.7 ± 2.8
	0.83	1.01	0.50	0.45	0.09	1.17	0.07	0.49	1.44
Frutafit agave fructan	85.7 ± 3.5	86.4 ± 2	86.8 ± 3	83.2 ± 1.5	78.7 ± 0.9	80.4 ± 4.3	84.3 ± 2.5	80.9 ± 1.1	83.3 ± 6.2
	2.07	1.17	1.75	0.91	0.55	2.66	1.49	0.66	3.69
Timothy grass (Cat. No. #171201)	14.4 ± 0.8	14.3 ± 0.1	14.8 ± 0.4	14.8 ± 0.5	15.6 ± 0.2	15.9 ± 1.1	15.2 ± 1	14.9 ± 0.8	15 ± 1.2
	2.93	0.50	1.50	1.61	0.51	3.58	3.20	2.84	4.01
Barley MAX	14.4 ± 0.2	14 ± 1.4	14.7 ± 0.7	14.3 ± 0.6	12.7 ± 0.6	—	14.6 ± 2.6	14.2 ± 2.3	14.1 ± 1.6
	0.60	4.92	2.46	2.27	2.51	—	8.84	7.94	5.77
Rye grass	11 ± 0.4	10.8 ± 0.3	10.4 ± 0.6	10.4 ± 0.1	10 ± 0.1	10.3 ± 0.2	10.3 ± 0.5	10.4 ± 0.3	10.4 ± 0.7
	1.73	1.25	2.98	0.46	0.57	0.74	2.27	1.63	3.25
Brett Brother swine feed	2 ± 0	2 ± 0	2 ± 0.1	1.9 ± 0.1	1.9 ± 0.1	2 ± 0.1	2.3 ± 0.1	2.1 ± 0.4	2.01 ± 0.26
	0.78	0.31	3.05	2.64	3.11	2.33	1.69	9.46	6.36
Barley flour (Cat. No. #60301a)	1.7 ± 0	1.7 ± 0.1	1.7 ± 0.1	1.5 ± 0.1	1.4 ± 0	1.5 ± 0.1	1.7 ± 0.3	1.7 ± 0	1.6 ± 0.2
	0.32	2.80	3.04	2.78	1.12	1.91	8.23	0.50	5.79
Purina GoCat dry cat food	1 ± 0	1 ± 0.2	1 ± 0	1 ± 0	0.9 ± 0	0.9 ± 0	1 ± 0.1	1 ± 0.1	0.98 ± 0.1
	1.73	9.18	0.16	0.78	1.54	1.17	4.55	3.65	4.84
TopSpec alfalfa	0.2 ± 0	0.2 ± 0	0.2 ± 0	0.2 ± 0	0.2 ± 0	0.2 ± 0	0.2 ± 0	0.2 ± 0.1	0.19 ± 0.03
	4.20	3.82	2.91	8.53	8.53	9.37	3.48	20.75	8.47

[a] Mean ± 2 SD.

[b] Laboratory 1 = Analyst 1.

[c] Laboratory 2 = Analyst 2.

exo-inulinase/endo-inulinase/endo-levanase) exhibit excellent stability for at least 4 years when stored as recommended (–20 and 4°C, respectively).

Conclusions

The fructan assay method described in this document is robust, reliable, and repeatable, and therefore suitable for measuring fructans in animal food and feed samples. The data presented in this report (Table 8) verifies and validates that this method is fit for the purpose intended and shows that this method meets all the parameter requirements set out in SMPR 2018.002 for fructans in animal feed, pet food, and ingredients.

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References

(1) Lewis, D.H. (1993) *New Phytologist* **124**, 583–594. doi:10.1111/j.1469-8137.1993.tb03848.x
(2) Pontis, H.G. (1990) in *Methods in Plant Biochemistry*, P.M. Dey & J.B. Harbourne (Eds), Academic Press, New York, NY, pp 353–369
(3) Bancal, P., Gibeaut, D.M., & Carpita, N.C. (1993) in *Science and Technology of Fructans*, M. Suzuki & N.J. Chatterton (Eds), CRC Press, London, United Kingdom, pp 83–118
(4) McCleary, B.V., & Blakeney, A.B. (1999) *Cereal Foods World* **44**, 398–406
(5) McCleary, B.V., Murphy, A., & Mugford, D.C. (2000) *J. AOAC Int.* **83**, 356–364
(6) McCleary, B.V., & Rossiter, P. (2004) *J. AOAC Int.* **87**, 707–717.
(7) Longland, A.C., Dhanoa, M.S., & Harris, P.A. (2012) *J. Sci. Food Agric.* **92**, 1878–1885. doi:10.1002/jsfa.5555