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# FRUCTAN
# ASSAY PROCEDURE

for the measurement of

**FRUCTO-OLIGOSACCHARIDES (FOS)**

and

**FRUCTAN POLYSACCHARIDE**

K-FRUC 03/14

***(with recombinant inulinases)***

(100 Assays per Kit)

**AOAC Method 999.03**
**AACC Method 32.32**
**Codex Type III Method**

## INTRODUCTION:

Fructans are defined as any compound where one or more fructosyl-fructose linkage constitutes a majority of the linkages (Lewis, 1993). This refers to polymeric material as well as oligomers as small as the disaccharide, inulobiose. Material included in this definition may or may not contain D-glucosyl substituents. The terms oligomer and polymer are used by fructan researchers to distinguish between materials which can be specifically characterised and those which cannot (Lewis, 1993). 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 main types: the inulin group, the levan group and the branched group. The inulin group consists of material that has mostly or exclusively the (2→1) fructosyl-fructose linkage. Levan is material which 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*).

Several procedures have been described for the measurement of fructan in plant material and food products. It is generally accepted that these are best measured after hydrolysis to D-fructose and D-glucose. This introduces the problem of independently removing, or measuring, sucrose, D-fructose and D-glucose. Pontis (1966) has reported the removal of sucrose, D-glucose and D-fructose by hydrolysing sucrose with a crystalline yeast invertase and destroying the resulting D-glucose and D-fructose as well as existing monosaccharides by boiling with sodium hydroxide. However, yeast invertase also hydrolyses lower degree of polymerisation (DP) fructo-oligosaccharides (FOS); at a concentration of 10 mg/mL, 1-kestose is hydrolysed at approx. 20% the rate of sucrose and 1,1-kestotetraose is hydrolysed at approx. 10% the rate of sucrose (McCleary and Blakeney, 1999).

An alternative approach (Quemener *et al.,* 1993) involves the use of capillary gas chromatography (CGC) or HPLC to analyse extracts of samples either untreated, or treated with amyloglucosidase or amyloglucosidase plus inulinase (fructanase). By measuring sucrose, D-fructose and D-glucose in the various samples, and with appropriate calculations, it is possible to get an estimate of free D-glucose and D-fructose, sucrose, starch and fructan. The possible interference of raffinose-series oligosaccharides (which may be present in some samples) was not considered. The crude fructanase enzyme preparation used in this work contains a very active α-galactosidase, and, consequently, any raffinose-series oligosaccharides present in the sample will also be hydrolysed to D-fructose and D-glucose (and D-galactose). Separate from the possible problems with raffinose-series oligosaccharides, this method is quite complex and requires the use of expensive equipment.

The currently described method (McCleary and Blakeney, 1999; McCleary and Rossiter, 2004) is specific for fructans, including those from chicory, dahlia, jerusalem artichoke, onion, wheat stems and leaves and agave. Of these, the highly branched (2→1),(2→6) fructan from agave (*Agavaceae*) is the most resistant, but even this is completely hydrolysed under the described assay conditions. Commercial fructan products such as Raftilose P-95® (partially hydrolysed chicory inulin) are underestimated by this method to an extent related to the degree of hydrolysis of the fructan (approx. 20%). The actual degree of underestimation is readily determined analytically if samples of the fructan/FOS ingredient are available (see Appendix A).

## PRINCIPLE:

Sucrose, maltose, maltodextrins and starch are hydrolysed to D-glucose and D-fructose. Sucrose is hydrolysed by a specific sucrase enzyme which has no action on lower degree of polymerisation (DP) FOS such as 1-kestose and 1,1-kestotetraose (McCleary and Blakeney, 1999). Starch and maltodextrins are hydrolysed to maltose and maltotriose by pullulanase and β-amylase, and these oligosaccharides are then hydrolysed to D-glucose by maltase. (1).

$$\text{(1)}\quad \text{Sucrose} + H_2O \xrightarrow{\text{(sucrase, pH 6.5, 40°C)}} \text{D-glucose} + \text{D-fructose}$$

$$\text{(2)}\quad \text{Starch} + \text{maltosaccharides} \xrightarrow{\text{(pullulanase, β-amylase, maltase, pH 6.5, 40°C)}} \text{D-glucose}$$

D-Glucose and D-fructose are reduced by sodium borohydride to the corresponding sugar alcohols, D-sorbitol and D-mannitol. In this reaction, the D-fructosyl residue at the reducing end of fructo-oligosaccharides in hydrolysed inulin preparations, is also reduced to the sugar alcohol. Native fructans and non-reducing FOS such as Neosugars® are not affected by this reaction.

$$\text{(3)}\quad \text{D-Glucose} + \text{D-fructose} \xrightarrow{\text{(sodium borohydride, room temperature)}} \text{D-sorbitol} + \text{D-mannitol} + \text{reduced fructo-oligosaccharides}$$

Fructan, FOS and borohydride reduced FOS are specifically hydrolysed by ultrapure (recombinant/affinity purified) *exo*-inulinase and *endo*-inulinase to D-glucose and D-fructose (McCleary and Blakeney, 1999). D-Mannitol is also present in the hydrolysate of reduced FOS (4).

$$\text{(4)}\quad \text{Fructan} + H_2O \xrightarrow{\text{(}\textit{exo}\text{-, and }\textit{endo}\text{-inulinases, pH 4.5, 40°C)}} \text{D-glucose} + \text{D-fructose}$$

D-Fructose and D-glucose derived from fructan is measured using the PAHBAH reducing sugar method (Lever, 1973). This method is simple

to use and the colour response for D-fructose and D-glucose is the same (5).

$$\text{(5) D-Glucose + D-fructose} \xrightarrow[\text{(PAHBAH reagent, 100°C, 6 min)}]{} \text{PAHBAH colour complex}$$

## SPECIFICITY, SENSITIVITY, LINEARITY AND PRECISION:

This assay was the subject of a successful interlaboratory evaluation under the auspices of AOAC International and AACC International (McCleary *et al.*, 1997). The assay is specific for fructans containing mostly or exclusively (2→1) fructosyl-fructose linkages (inulin) and those with both (2→1) and (2→6) fructosyl-fructose linkages in significant amounts (e.g. those from onion, Gramineae and Agave). Borohydride reduced FOS are hydrolysed releasing D-mannitol from the "reducing end" terminus. Sucrose, maltose, maltodextrins and starch are "removed" by enzymic hydrolysis followed by borohydride reduction, before fructan hydrolysis. Galactosyl-sucrose oligosaccharides, if present, must be enzymatically removed. See Controls and Precautions 8, page 7. The complete absence of β-glucanase and β-glucosidase in the fructanase preparation means that trace levels of fructan in cereal grain can be measured with no contribution to reducing sugar level by partial hydrolysis of mixed-linkage β-glucan by β-glucanases.

The smallest differentiating absorbance for the assay is 0.010 absorbance units. This corresponds to 12.8 µg/mL of D-glucose plus D-fructose in the sample extract being analysed. The detection limit is 25.6 µg of D-fructose and/or D-glucose per mL of sample extract, which is derived from an absorbance difference in the assay of 0.02.

The assay is linear over the range of 2.3 to 55 µg of D-fructose or D-glucose per assay. In duplicate determinations using one sample solution, an absorbance difference of 0.005 to 0.010 may occur, this corresponds to a D-fructose or D-glucose level of 0.023-0.046 µg per assay.

## INTERFERENCE:

Interfering substances in the sample being analysed can be identified by including a fructan internal standard. Quantitative recovery of this standard would be expected. Losses in sample handling and extraction are identified by performing recovery experiments, i.e. by adding D-fructose or D-glucose to the sample in the initial extraction steps.

## SAFETY:

The general safety measures that apply to all chemical substances should be adhered to.

For more information regarding the safe usage and handling of this product please refer to the associated SDS that is available from the Megazyme website.

## KIT CONTENTS:

Kits suitable for performing 100 determinations of fructan are available from Megazyme. The kits contain the full assay method plus:

**Bottle 1.** **Sucrase** plus β-amylase, pullulanase and maltase as a freeze-dried powder.
Stable for > 5 years at -20°C.

**Bottle 2.** **Fructanase.** Recombinant *exo*-inulinase and recombinant *endo*-inulinanase as a freeze-dried powder.
Stable for > 5 years at -20°C.

**Bottle 3.** **Fructan Control Flour.** Fructan freeze-dried in the presence of α-cellulose.
Stable for > 5 years stored dry at room temperature.

**Bottle 4.** **Sucrose Control Flour.** Sucrose freeze-dried in the presence of α-cellulose.
Stable for > 5 years stored dry at room temperature.

**Bottle 5.** **D-Fructose Standard Solution** (1.5 mg/mL) in 0.2% (w/v) benzoic acid.
Stable for > 5 years at room temperature.

## PREPARATION OF ENZYMES:

**1.** Dissolve the contents of bottle 1 in 22 mL of Buffer 1 [sodium maleate (100 mM, pH 6.5)] (Enzyme Solution A). Divide into aliquots of appropriate volume and store at -20°C.
Stable in polypropylene containers for > 5 years at -20°C.

**2.** Dissolve the contents of vial 2 in 22 mL of Buffer 2 [sodium acetate (0.1 M, pH 4.5)] (Enzyme Solution B). Divide into aliquots of appropriate volume and store at -20°C.
Stable in polypropylene containers for > 5 years at -20°C.

**3, 4 & 5.** Use the contents of bottles 3, 4 and 5 as supplied.
Stable for > 5 years at room temperature.

## BUFFERS (not supplied):

**Buffer 1:** **Sodium maleate buffer** (100 mM, pH 6.5)
Dissolve maleic acid (11.6 g, Sigma cat. no. M-0375) in 900 mL of distilled water and adjust the pH to 6.5 with sodium hydroxide solution (2 M). Adjust volume to 1 litre.
Stable for > 3 months at 4°C.

**Buffer 2:** **Sodium acetate buffer** (100 mM, pH 4.5)
Add glacial acetic acid (5.8 mL) to 900 mL of distilled water. Adjust to pH 4.5 using 1 M sodium hydroxide. Adjust the volume to 1 litre.
Stable for > 3 months at 4°C.

## REAGENTS (not supplied):

1. **PAHBAH Reducing Sugar Assay Reagent**

   **Solution A**. Add 10 g of *p*-hydroxybenzoic acid hydrazide (Sigma cat. no. H-9882) (PAHBAH) to 60 mL of distilled water in a 250 mL beaker on a magnetic stirrer. Stir the slurry and add 10 mL of concentrated hydrochloric acid. Adjust the volume of the solution to 200 mL with distilled water and store at room temperature. Stable for ~ 2 years.

   **Solution B**. Add 24.9 g of trisodium citrate dihydrate to 500 mL of distilled water and stir to dissolve. Add 2.2 g of calcium chloride dihydrate and dissolve. Add 40.0 g of sodium hydroxide and dissolve with stirring (the solution may be milky, but will clarify when diluted to 2 litres). Adjust the volume to 2 litres and store the solution at room temperature. Stable for ~ 2 years.

   **PAHBAH Working Reagent.** Immediately before use, add 20 mL of Solution A to 180 mL of Solution B and mix thoroughly. The mixed solution stored on ice is stable for ~ 4 hours.

2. **Sodium hydroxide (50 mM)**

   Dissolve 2.0 g of sodium hydroxide in 900 mL of distilled water. Adjust the volume to 1 litre. Store at room temperature.

3. **Alkaline borohydride (10 mg/mL sodium borohydride in 50 mM sodium hydroxide)**

   Accurately weigh approx. 50 mg of sodium borohydride (Sigma cat. no. S-9125) into polypropylene containers (10 mL volume with screw cap). Record the exact weight on the tubes (approx. 10 for convenience), seal the tubes and store them in a desiccator for future use.

   Immediately before use, dissolve the sodium borohydride (at 10 mg/mL) in 50 mM sodium hydroxide. This solution is stable for 4-5 hours at room temperature.

4. **Acetic acid (200 mM)**

   Add 11.6 mL of glacial acetic acid to 600 mL of distilled water and adjust the volume to 1 litre. Store at room temperature.

## EQUIPMENT (RECOMMENDED):

1. Glass test tubes (round bottomed; 16 x 100 mm and 18 x 150 mm).
2. Pyrex beakers (100 and 200 mL capacity).
3. Volumetric flasks (50 and 100 mL capacity).
4. Micro-pipettors, e.g. Gilson Pipetman® (100 µL and 200 µL).

5. Positive displacement pipettor, e.g. Eppendorf Multipette®
   - with 5.0 mL Combitip® (to dispense 0.2 mL aliquots of sucrase mixture and 0.1 mL aliquots of fructanase, and other solutions and buffers).
   - with 50 mL Combitip® (to dispense 5.0 mL aliquots of PAHBAH Working Reagent).
6. Analytical balance.
7. Spectrophotometer set at 410 nm.
8. Vortex mixer (e.g. IKA® Yellowline Test Tube Shaker TTS2).
9. Thermostated water bath (set at 40°C).
10. Boiling water bath.
11. Hot-plate magnetic stirrer.
12. Bench centrifuge (capable of 1,000 *g*) or filter funnels with Whatman No. 1 (9 cm) filter papers.
13. Stop watch.

## CONTROLS AND PRECAUTIONS:

1. The time of incubation at 100°C with PAHBAH reagent is critical and should be timed with a stop watch.
2. With each set of determinations, reagent blanks and D-fructose controls should be included and analysed concurrently.
   a) The reagent blank consists of 0.3 mL of 100 mM sodium acetate buffer (Buffer 2) + 5.0 mL of PAHBAH Working Reagent.
   b) To prepare the D-fructose standard, 0.2 mL of D-fructose standard solution (1.5 mg/mL) is added to 0.9 mL of Buffer 2 [100 mM sodium acetate (pH 4.5)] and mixed thoroughly. Aliquots (0.2 mL) of this solution (containing 54.5 µg of D-fructose) are dispensed, in quadruplicate, into glass test tubes (16 x 100 mm). Buffer 2 (0.1 mL) is added to each tube plus 5.0 mL PAHBAH Working Reagent (immediately before incubation in the boiling water bath).
3. With each set of determinations a **fructan/cellulose control powder** [12-50% (w/w) fructan format] is included. The fructan content of this powder is given on the vial label.
4. The **sucrose/cellulose control** powder should be analysed with each new lot of reagents [0-12% (w/w) fructan format]. If the sucrase treatment step is completely effective, the determined fructan value should be approx. 0.2% (w/w). If the sucrase is not effective, the determined value will reflect the sucrose content of the control sucrose/cellulose powder [about 10% (w/w); see vial label].

5. D-Fructose **controls** (quadruplicate) and **reagent blank** solutions (duplicate) are run with each batch of samples and are incubated in the boiling water bath at the same time as the samples.
6. The effectiveness of borohydride reduction can be checked using D-fructose standard solution (0.2 mL, 1.5 mg/mL) and proceeding from **Step B.1.** (page 8) of the assay procedure. Treatment with fructanase enzyme (**Step C.2.**) is replaced with addition of acetate buffer (0.1 mL, 0.1 M, pH 4.5). The solution should be colourless following incubation with PAHBAH Working Reagent.
7. The PAHBAH Working Reagent should be used fresh or stored on ice for no more than 4 hours.
8. If the sample being analysed contains **galactosyl-sucrose oligosaccharides**, these can be removed by incubation with *A. niger* α-galactosidase (Megazyme cat. no. E-AGLANP). Add 50 µL of α-galactosidase (200 U/mL) in 50 mM sodium acetate buffer (pH 4.5) to 0.2 mL of solution to be analysed and incubate for 30 min at 40°C before addition of the Sucrase/Amylase working mixture (Enzymes Solution 1). This enzyme gives complete hydrolysis of D-galactose from galactosyl-sucrose oligosaccharides.

## ASSAY PROCEDURE:

### A. Fructan Extraction:

Dry samples are milled to pass a 0.5 mm screen. Solid fatty samples (e.g. chocolate) are cut into fine shavings with a sharp knife; soft food products (e.g. spreads) are analysed without further preparation. All samples should be at room temperature before they are weighed.

**Samples containing 0-12% (w/w) fructan**

1. Accurately weigh 1.0 g of the sample into a dry pyrex beaker (200 mL capacity) and add 80 mL of hot distilled water (~ 80°C). Place the beaker on a hot-plate/magnetic stirrer and stir with heating (at ~ 80°C) for 15 min (i.e. until the sample is completely dispersed).
2. Allow the solution to cool to room temperature and then quantitatively transfer it to a 100 mL volumetric flask. Adjust the volume to the mark with distilled water and mix the contents thoroughly.

**Samples containing 12-50% (w/w) fructan**

1. Accurately weigh approx. 100 mg of the sample into a dry pyrex beaker (100 mL capacity) and add 40 mL of hot distilled water (~ 80°C). Place the beaker on a hot-plate, magnetic stirrer and stir with heating (at ~ 80°C) for 15 min (i.e. until the sample is completely dispersed).

2. Allow the solution to cool to room temperature and then quantitatively transfer it to a 50 mL volumetric flask. Adjust the volume to the mark with distilled water and mix the contents thoroughly.

> **Note:** For samples containing 50-100% (w/w) fructan, the volume is adjusted to 100 mL in a 100 mL volumetric flask.

**Further treatment of extracts:**

3. Filter an aliquot of the solution through a Whatman No. 1 (9 cm) filter circle and analyse immediately (this solution may be slightly turbid, depending on the nature of the sample extracted). If this solution is stored for several hours at low temperature before analysis, the fructan may tend to precipitate from solution. In such cases, the solution should be reheated to ~ 80°C and allowed to cool to room temperature before samples are removed for analysis.

## B. Removal of Sucrose, Starch and Reducing Sugars:

1. Accurately dispense a 0.2 mL aliquot of the solution to be analysed (containing approx. 0.1-1.0 mg/mL of fructan) into the bottom of a glass test tube (16 x 100 mm).
2. Add 0.2 mL of diluted Sucrase/Amylase solution (Enzyme Solution A) and incubate the tube at 40°C for 30 min.

> **NOTE:** If samples contain very high levels of sucrose and maltodextrins (e.g. baby milk formulations), increase this incubation time to 60 min to ensure complete hydrolysis of these oligosaccharides.

3. Add 0.2 mL of Reagent 3 (alkaline borohydride solution) to the tube, stir vigorously and incubate at 40°C for 30 min to effect complete reduction of reducing-sugars to sugar alcohols.
4. Add 0.5 mL of Reagent 4 (200 mM acetic acid) to the tube with vigorous stirring on a vortex mixer. **A vigorous effervescence should be observed** (this treatment removes excess borohydride and adjusts the pH to approx. 4.5). This is termed **Solution S**.

## C. Hydrolysis and Measurement of Fructan:

1. Accurately and carefully transfer 0.2 mL aliquots of **Solution S** into the bottom of three (3) glass test-tubes (16 x 100 mm).
2. Add 0.1 mL of fructanase solution (Enzyme Solution B) to two of these tubes (**samples**) and 0.1 mL of 0.1 M sodium acetate buffer to the third (**sample blank**).

3. Incubate the tubes at 40°C for 30 min to effect complete hydrolysis of fructan to D-fructose and D-glucose. Seal the tubes with Parafilm® during incubation.
4. Add 5.0 mL of PAHBAH Working Reagent to all tubes [**samples, sample blanks**, the **D-fructose standard** (see Controls and Precautions 2.b), **reagent blank** (Controls and Precautions 2.a) and the extract of the **fructan/cellulose control sample**] and incubate in a boiling water bath for exactly 6 min.
5. Remove the tubes from the boiling-water bath and immediately place them in cold water (18-20°C) for approx. 5 min.
6. Measure the absorbance of all solutions at 410 nm against the reagent blank.

   Measure the absorbance values as soon as possible after cooling the tubes. **The PAHBAH colour complex will fade with time.**

$$= \Delta_A \times F \times 5 \times V \times \frac{1.1}{0.2} \times \frac{100}{W} \times \frac{1}{1000} \times \frac{162}{180}$$

$$= \Delta_A \times F \times \frac{V}{W} \times 2.48$$

## CALCULATIONS:

### Fructan (% w/w as is):

where:

$\Delta_A$ = sample absorbance - sample blank absorbance (both read against the reagent blank)

F = factor to convert absorbance values to µg of D-fructose
= (54.5 µg D-fructose)/(absorbance for 54.5 µg D-fructose)

5 = factor to convert from 0.2 mL as assayed to 1.0 mL

V = volume (mL) of extractant used (i.e. 50 or 100 mL)

$\frac{1.1}{0.2}$ = 0.2 mL was taken from 1.1 mL of enzyme digest for analysis

W = weight (mg) of sample extracted

$\frac{100}{W}$ = factor to express fructan as a percentage of flour weight

$\frac{1}{1000}$ = factor to convert from µg to mg

$\frac{162}{180}$ = factor to convert from free D-fructose, as determined, to anhydrofructose (and anhydroglucose), as occurs in fructan

**NOTE:** These calculations can be simplified by using the Megazyme ***Mega-Calc***™, downloadable from where the product appears in the Megazyme web site (www.megazyme.com).

## APPENDIX:

### A. Determination of the Extent of Underestimation of Fructan Content as a Consequence of Borohydride Reduction of Hydrolysed Inulin:

1. Accurately weigh approx. 100 mg of the sample into a dry pyrex beaker (100 mL capacity) and add 80 mL of hot distilled water (~ 80°C). Place the beaker on a hot-plate, magnetic stirrer and stir with heating (at ~ 80°C) for 15 min (i.e. until the sample is completely dispersed).
2. Allow the solution to cool to room temperature and then quantitatively transfer it to a 100 mL volumetric flask. Adjust the volume to the mark with distilled water and mix the contents thoroughly.
3. Proceed with the standard K-FRUC assay procedure from step A.3. to Step B.1. on page 8, **then:**
4. Accurately dispense two (2) 0.2 mL aliquots of the solution to be analysed (containing approx. 0.1-1.0 mg/mL of fructan) into the bottom of glass test tubes (16 x 100 mm).
5. **With one of these samples**, proceed with the standard K-FRUC assay protocol and determine the fructan content in "borohydride reduced samples (R)".
6. **With the second sample**, add 0.2 mL of diluted Sucrase/ Amylase solution (Enzyme Solution A) and incubate the tube at 40°C for 30 min.
7. Add 0.7 mL of Buffer 2 (100 mM sodium acetate buffer, pH 4.5) to the tube with vigorous stirring on a vortex mixer. This is termed **Solution S**.

### C. Hydrolysis and Measurement of Fructan:

1. Accurately and carefully transfer 0.2 mL aliquots of **Solution S** into the bottom of four (4) glass test-tubes (16 x 100 mm).
2. Add 0.1 mL of fructanase solution (**Enzyme Solution 2**) to two of these tubes (**samples**) and 0.1 mL of 100 mM sodium acetate buffer to the other two (**sample blanks**).
3. Proceed with fructan determination according to the standard K-FRUC from step C.3. (page 9) and determine the fructan content in the "non-borohydride reduced samples (NR)".

**Percentage recovery** of fructan in the standard procedure with borohydride reduction:

**= R / NR x 100.**

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