

# Megazyme

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## FRUCTAN ASSAY PROCEDURE

FRUC 10/2002

FOR THE MEASUREMENT OF  
OLIGOFRACTAN AND  
FRUCTAN POLYSACCHARIDE  
SEPARATE FROM SUCROSE AND  
REDUCING SUGARS

**AOAC Method 999.03**  
**AACC Method 32.32**



## INTRODUCTION:

Fructans are defined as any compound where one or more fructosyl-fructose linkages constitute 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 attached glucose. 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.

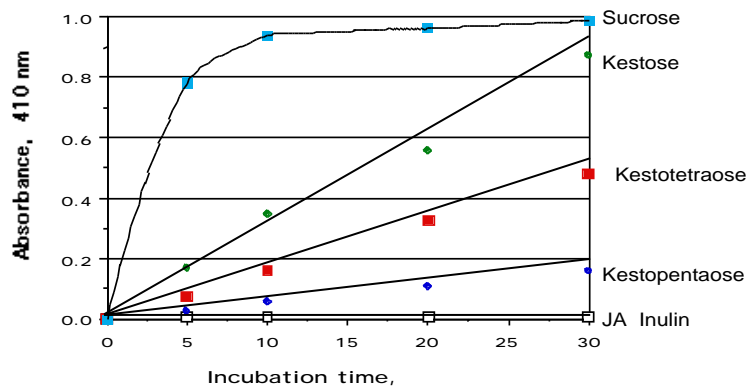
Fructans differ in molecular structure and in molecular weight. They may be classified in three main types (Pontis,1990): the inulin group, the phlein group and the branched group. The inulin group consists of material that has mostly or exclusively the (2-1) fructosyl-fructose linkage. Phlein (or levan) 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 fructose (and glucose). This introduces the problem of independently removing, or measuring, sucrose, fructose and glucose. Pontis (1966) has reported the removal of sucrose, glucose and fructose by hydrolysing sucrose with a crystalline yeast invertase and destroying the resulting glucose and fructose as well as existing monosaccharides by boiling with sodium hydroxide. It was claimed that the action of invertase on the lower fructan members of the inulin series is slow and can be rendered insignificant by judicious selection of the incubation conditions. In testing currently available pure yeast invertases, we have found that it is extremely difficult, if not impossible to achieve these conditions, as shown in **Figure 1**.

In this figure, the relative rates of hydrolysis of sucrose, 1-kestose, 1,1-kestotetraose, 1,1,1-kestopentaose and jerusalem artichoke inulin (polysaccharide) by yeast invertase are compared. It is evident that 1-kestose is hydrolysed at approximately 20% the rate for sucrose, and 1,1-kestotetraose is hydrolysed at ~10% the rate for sucrose.

An alternative approach (Quemener *et al.*, 1993) involves the use of capillary gas chromatography (CGC) or HPLC to analyse extracts of samples either non treated, or treated with amyloglucosidase or amyloglucosidase plus inulinase (fructanase). By measuring sucrose, fructose and glucose in the various samples, and with appropriate calculations, it is possible to get an estimate of free glucose and 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  $\beta$ -galactosidase, consequently, any raffinose-series oligosaccharides present in the sample will also be hydrolysed to fructose and glucose (and galactose). Separate from the possible problems with raffinose-series oligosaccharides, this method is quite complex, and requires the use of expensive equipment.

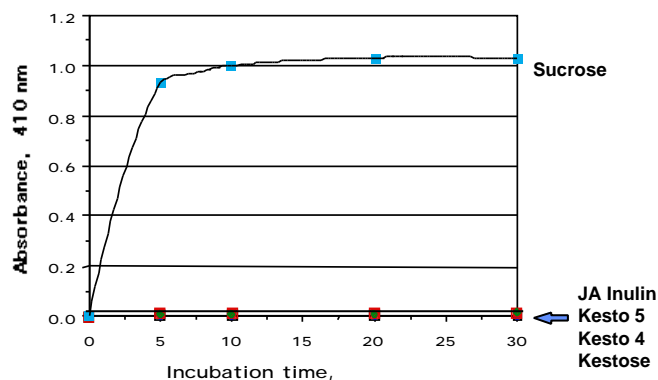


**Figure 1. Hydrolysis of sucrose and fructans by yeast invertase.**

Sugar compound (0.2 mL, 50  $\mu$ g) was incubated with invertase (2 units) in sodium acetate buffer (100 mM, pH 4.5) at 40°C. Reaction was terminated at various time intervals with PAHBAH working reagent and colour developed. Jerusalem artichoke inulin (JA Inulin), 1-kestose (Kesto 3); 1,1-kestotetraose (Kesto 4); 1,1,1-kestopentaose (Kesto 5); and sucrose.

In contrast, the method described in this booklet, is easy to perform, uses standard laboratory equipment, and is accurate, reproducible and specific. This procedure employs highly purified and specific enzymes to hydrolyse sucrose, starch and fructans. The **sucrase** enzyme used in this method rapidly hydrolyses sucrose but has negligible activity on 1-kestose and other fructo-oligosaccharides (McCleary and Blakeney, unpublished data) (**Figure 2**). At substrate concentrations of 10 mg/mL, the relative rate of hydrolysis of sucrose and 1-kestose is 3,800:1.

The Megazyme method is applicable to the measurement of fructan in plant materials and food mixtures. Hydrolysis of fructans from chicory (polymeric fraction), onion and wheat leaves is shown in the thin layer chromatography (Tlc) results in **Figure 3**. Fructan [5 g/100 mL in 10 mM sodium acetate buffer (pH 4.5)] was incubated with 4,000 U fructanase (*exo*-inulinase) at 40°C. Aliquots were removed at 0, 5, 20 and 60 mins, incubated at 100°C to inactivate the enzyme and analysed by Tlc, and by the PAHBAH reducing sugar method. Reducing sugar values were calculated as a percentage of total carbohydrate, and are shown in Figure 3 (top). Samples were also analysed by Bio-Gel P-2 chromatography (**Figure 4**).

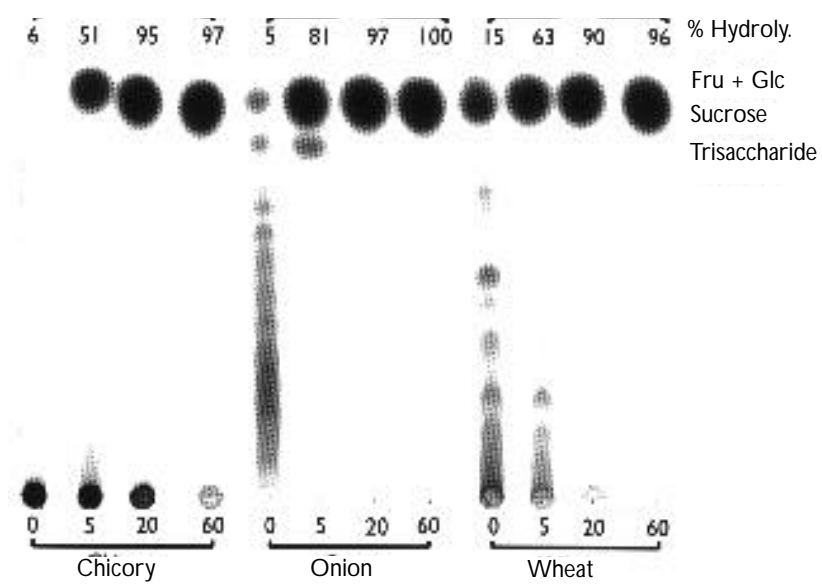


**Figure 2. Hydrolysis of sucrose and fructans by sucrase.**

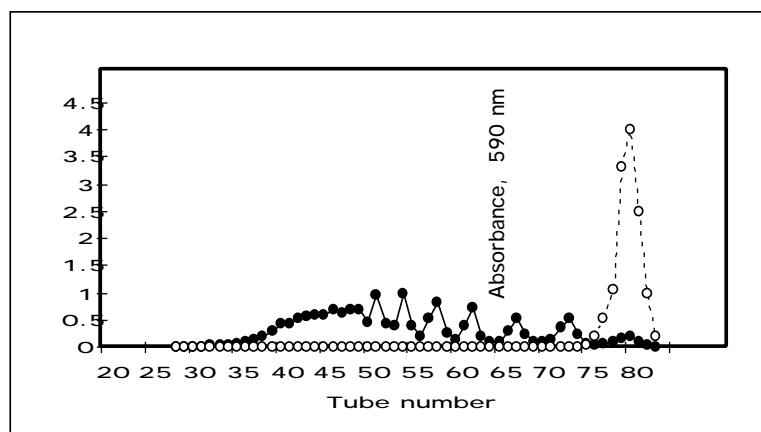
Sugar compound (0.2 mL, 50 µg) was incubated with sucrase (1 unit) in sodium maleate buffer (100 mM, pH 6.5) at 40°C. Reaction was terminated at various time intervals with PAHBAH working reagent and colour developed.

**PRINCIPLE:**

Sucrose is hydrolysed to glucose and fructose using a specific **sucrase**. Concurrently, starch and maltosaccharides (if present in the sample) are hydrolysed to glucose by the combined action of highly purified – amylase, pullulanase and maltase. These reducing-sugars are then reduced to the sugar alcohols by treatment with alkaline borohydride. The solution is neutralised and excess borohydride is removed by treatment with dilute acetic acid. The fructan is hydrolysed to fructose and glucose with purified fructanase (*exo-inulinase*) and the reducing sugars produced (fructose and glucose) are measured with the PAHBAH reducing-sugar method. This method is simple to use and the colour response with fructose and glucose is the same. For samples containing raffinose-series oligosaccharides, we recommend the inclusion of *A.niger* -galactosidase in the initial incubation step. The released monosaccharides will then be removed in the alkaline borohydride step.



**Figure 3.** Thin layer chromatography of the sugars produced on hydrolysis of chicory, onion and wheat fructans by *exo*-inulinase (conditions as described in the text). Samples were taken at 0,5,20 and 60 min for chromatography. Tic plates were developed once with *n*-propanol-ethanol-water (7:1:2).



**Figure 4.** Bio-Gel P-2 chromatography of the sugars produced on hydrolysis of onion fructan by *exo*-inulinase (incubation conditions as described in the text). Column eluates were analysed by the phenol-sulphuric acid procedure. Incubated for 0 min (●) and 60 min (○).

#### KIT CONTENTS:

Kits contain sufficient reagent for 100 determinations of fructan, i.e. they contain the full assay method plus:

1. **Sucrase** (100 U) plus  $\beta$ -amylase (*B. cereus*, 500 U), pullulanase (*K.pneumoniae*, 100 U) and maltase (yeast, 1,000 U) as a freeze-dried powder. Store desiccated at  $-20^{\circ}\text{C}$ .
2. **Fructanase**. Highly purified *exo*-inulinase (10,000 U) and *endo*-inulinase (100 U) as a freeze-dried powder. Store desiccated at  $-20^{\circ}\text{C}$ .
3. **Fructan Control Flour**. Dahlia fructan freeze-dried in the presence of  $\alpha$ -cellulose. Store dry at room temperature.
4. **Sucrose Control Flour**. Sucrose freeze-dried in the presence of  $\alpha$ -cellulose. Store dry at room temperature.
5. **Fructose Standard Solution** (1.5 mg/mL) in 0.2% benzoic acid. Store at room temperature.

#### ENCLOSED ENZYMES:

- (A) **Sucrase/ $\beta$ -Amylase/Pullulanase/Maltase** (freeze-dried powder)  
Dissolve the entire contents of the vial in 22 mL of **Buffer 1** [sodium maleate (0.1 M, pH 6.5)]. Divide into aliquots of appropriate volume and store frozen in polypropylene containers between use.
- (B) **Fructanase** (freeze-dried powder)  
Dissolve the contents of the vial in 22 mL of **Buffer 2** [sodium acetate (0.1 M, pH 4.5)]. Divide into aliquots of appropriate volume and store frozen in polypropylene containers between use.

#### ENCLOSED STANDARD:

Fructose Standard Solution (1.5 mg/mL in 0.2% benzoic acid).

#### ENCLOSED CONTROL FLOURS:

1. A fructan/cellulose powder of determined fructan content.
2. A sucrose/cellulose powder of determined sucrose content.

#### BUFFERS (not enclosed):

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 L. Store at  $4^{\circ}\text{C}$ .
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.

Store at 4°C.

**REAGENTS** (not enclosed):

**1. PAHBAH Reducing Sugar Assay Reagent**

**Solution A.** *p*-Hydroxybenzoic acid hydrazide (Sigma Cat. No. H-9882) (PAHBAH) (10 grams) is added to 60 mL of water in a 250mL beaker on a magnetic stirrer. The slurry is stirred and 10mL of concentrated hydrochloric acid is added. The solution is adjusted to 200 mL with distilled water and stored at room temperature.

**Stable for at least 2 years.**

**Solution B.** Trisodium citrate (24.9 g) is added to 500 mL of distilled water and stirred to dissolve. Calcium chloride dihydrate (2.20 g) is added and dissolved. Sodium hydroxide (40.0 g) is then added and dissolved with stirring. The solution may be milky, but will clarify on dilution to 2 litres. The volume is adjusted to 2 litres and the solution is stored at room temperature. **Stable for at least 2 years.**

**PAHBAH Working Reagent.** Immediately before use, add 20mL of Reagent A to 180 mL of Reagent B and mix thoroughly. This solution should be stored on ice and is stable for about 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)**

Into polypropylene containers (10 mL volume with screw cap) accurately weigh approximately 50 mg of sodium borohydride (Sigma Cat. No. S-9125). Record the weight on the tubes (approximately 10 for convenience), seal the tubes and store them in a desiccator for future use.

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

**4. Acetic acid (100 mM)**

Glacial acetic acid (5.8 mL) is added to distilled water and the volume is adjusted to 1 litre. Store at room temperature.

**EQUIPMENT (RECOMMENDED):**

1. Glass test tubes (round bottomed; 16 x 100 mm and 18 x 150 mm) and pyrex beakers (100 and 200 mL capacity).

2. Volumetric flasks (50 and 100 mL capacity).
3. Micro-pipettors, e.g. Gilson Pipetman<sup>®</sup> 200  $\mu$ L, and 100  $\mu$ L.
4. Positive displacement pipettor e.g. Eppendorf Multipette<sup>®</sup>
  - with 5.0 mL Combitip<sup>®</sup> (to dispense 0.2 mL aliquots of sucrose mixture and 0.1 mL aliquots of fructanase, and other solutions and buffers).
  - with 50 mL Combitip<sup>®</sup> (to dispense 5.0 mL aliquots of PAHBAH Working Reagent).
5. Analytical balance.
6. Spectrophotometer set at 410 nm.
7. Vortex mixer (we recommend the Thermolyne Maxi-Mix II).
8. Thermostated water bath (set at 40.0°C).
9. Boiling water bath.
10. Hot-plate magnetic stirrer.
11. Bench centrifuge (capable of speeds of 1,000g) or filter funnels with Whatman No. 1 (9 cm) filter papers.
12. Stop clock.

#### CONTROLS AND PRECAUTIONS:

1. The time of incubation at 100°C with PAHBAH reagent is critical and should be timed with a stop watch. After the 6 min incubation time, the rack of tubes should be immediately immersed in a cold-water bath (about 5-10°C) and left there for 5-10 min to cool. The absorbances of the reaction solutions should be measured within 10 min.
2. With each set of determinations, reagent blanks and 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 **PAHBAH Working Reagent**.
  - b) To prepare the fructose standard, 0.2 mL of **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  $\mu$ g of 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** 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. If the sucrase treatment step is completely effective, the determined fructan value should be about 0.2%. If the sucrase is not effective, the determined value will reflect the sucrose content of the control sucrose/cellulose powder (about 10%; see vial label).
5. 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 fructose standard solution (0.2 mL, 1.5 mg/mL) and proceeding from **Step a** of the assay procedure. Treatment with fructanase enzyme (**Step i**) is replaced with addition of acetate buffer (0.1 mL, 100 mM, pH 4.5). The solution should be colourless following incubation with PAHBAH Working Reagent
7. If the PAHBAH Working Reagent is stored too long before use, a **turbidity** will develop when the tubes are incubated in the boiling-water bath. This will not occur if fresh solution is used or if the solution, once prepared, is stored on ice (for no longer than 4 hours).
8. If the sample being analysed contains **raffinose-series oligosaccharides**, these can be removed by treatment with *A.niger* -galactosidase (Megazyme Cat. No. E-AGLAN) which is added to the Sucrase/Amylase working mixture (Enzyme A) at a concentration of 5 Units/mL. This enzyme, together with the sucrase, gives complete hydrolysis of raffinose-series oligosaccharides to monosaccharides which are reduced with the borohydride.

## 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% fructan

1. The sample (1.0 g) is accurately weighed into a dry pyrex beaker (200 mL capacity) and is treated with 80 mL of hot distilled water (~80°C). The beaker is placed on a hot-plate, magnetic stirrer and stirred and heated (at ~80°C) for 15 min (i.e. until the sample is completely dispersed).
2. The solution is allowed to cool to room temperature and then quantitatively transferred to a 100 mL volumetric flask and the volume adjusted to the mark with distilled water. The contents are mixed thoroughly.

### Samples containing 12-50% fructan

1. The sample (approx. 100 mg, weighed accurately) is weighed into a dry pyrex beaker (100 mL capacity) and is treated with 40 mL of hot distilled water (~80°C). The beaker is placed on a hot-plate, magnetic-stirrer and stirred and heated (at ~80°C) for 15 min (i.e. until the sample is completely dispersed).
2. The solution is allowed to cool to room temperature and then quantitatively transferred to a 50 mL volumetric flask and the volume is adjusted to the mark with distilled water. The contents are mixed thoroughly.

**Note:** For samples containing 50-100% fructan, the volume is adjusted to 100 mL.

### Further treatment of extracts:

3. An aliquot of the solution is filtered through a Whatman No. 1 (9 cm) filter circle and is analysed 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:

- a. Aliquots (0.2 mL) of solutions to be analysed (containing approximately 0.1 to 1.0 mg/mL of fructan) are accurately dispensed into the bottoms of glass test-tubes (16 x 100 mm).
- b. To each tube is added an aliquot (0.2 mL) of diluted Sucrase/ Amylase solution (**Enzyme A**) and the tubes are incubated at 40°C for 30 min.
- c. **Reagent 3** (alkaline borohydride solution; 0.2 mL) is added to each tube. The tube is stirred vigorously and stored at 40°C for 30 min to effect complete reduction of reducing-sugars to sugar alcohols.
- d. **Reagent 4** [acetic acid (100 mM); 0.5 mL] is added to each 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 A**.

### C. Hydrolysis and Measurement of Fructan:

- i. Aliquots (0.2 mL) of **Solution A** are accurately and carefully dispensed (in duplicate) into the bottoms of glass test-tubes (16x100 mm).
- ii. Fructanase solution (**Enzyme 2**) (0.1 mL) is added to each test-tube and the contents are mixed on a vortex stirrer.

- iii. Tubes are incubated at 40°C for 20 min to effect complete hydrolysis of fructan to fructose and glucose.
- iv. All tubes, including the **fructose standard** (Controls and Precautions 2 b), **reagent blank** (Controls and Precautions 2 a) and the extract of the **fructan/cellulose control sample**, are treated with PAHBAH Working Reagent (5.0 mL) **and** incubated in a boiling water bath for exactly 6 min.
- v. The tubes are removed from the boiling-water bath and immediately placed in cold water (18-20°C) for about 5 min.
- vi. The absorbance of all solutions are then measured at 410 nm against the reagent blank.  
Absorbance values should be measured as soon as possible after cooling the tubes. **The PAHBAH colour complex will fade with time.**

#### CALCULATIONS:

Fructan (% w/w as is):

$$\begin{aligned}
 &= E \times F \times 5 \times V \times \frac{1.1}{0.2} \times \frac{100}{W} \times \frac{1}{1000} \times \frac{162}{180} \\
 &= E \times F \times \frac{V}{W} \times 2.48
 \end{aligned}$$

where:

- E = PAHBAH absorbance of reaction solutions (0.2 mL) read against the reagent blank.
- F = factor to convert absorbance values to µg fructose.  
= (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.
- V = volume (mL) of extractant used (i.e. 100 or 50 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 fructose, as determined, to anhydrofructose (and anhydroglucose), as occurs in fructan.

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