

Megazyme

FRUCTAN ASSAY PROCEDURE

for the measurement of

**FRUCTO-OLIGOSACCHARIDES
(FOS)**

and

FRUCTAN POLYSACCHARIDE

K-FRUC 5/2008

(100 Assays per Kit)

AOAC Method 999.03

AACC Method 32.32



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. 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 approx. 20 % the rate for sucrose, and 1,1-kestotetraose is hydrolysed at approx. 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 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.

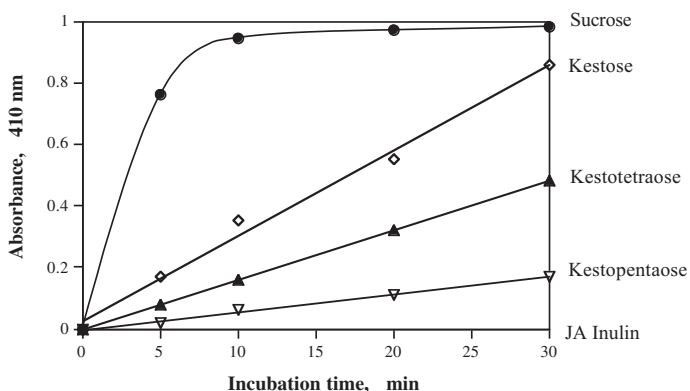


Figure 1. Hydrolysis of sucrose and fructans by yeast invertase.

Sugar compound (0.2 mL, 50 μ 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. The substrates used were Jerusalem artichoke inulin (JA Inulin), l-kestose; l,l-kestotetraose, l,l,l-kestopentaose, 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 l-kestose and other fructo-oligosaccharides (McCleary and Blakeney, 1999) (Figure 2). At substrate concentrations of 10 mg/mL, the relative rate of hydrolysis of sucrose and l-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 of fructanase (exo-inulinase) at 40°C. Aliquots were removed at 0, 5, 20 and 60 min, 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).

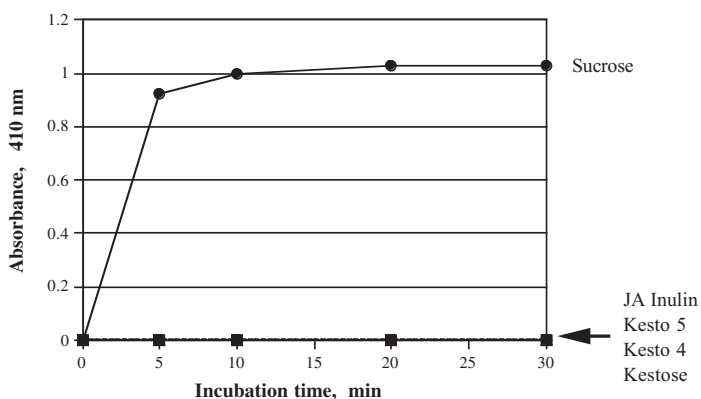


Figure 2. Hydrolysis of sucrose and fructans by sucrose.

Sugar compound (0.2 mL, 50 μ g) was incubated with sucrose (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. Kesto 4 (1,1-kestotetraose), Kesto 5 (1,1,1-kestopentaose).

PRINCIPLE:

Sucrose is hydrolysed to D-fructose and D-glucose using a specific sucrose enzyme. Concurrently, starch and maltosaccharides (if present in the sample) are hydrolysed to D-glucose by the combined action of highly purified β -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 D-fructose and D-glucose with purified fructanase (exo-inulinase) and the reducing sugars produced (D-fructose and D-glucose) are measured with the PAHBAH reducing-sugar method. This method is simple to use and the colour response with D-fructose and D-glucose is the same. For samples containing galactosyl-sucrose oligosaccharides (e.g. raffinose), we recommend incubation with *A. niger* α -galactosidase before addition of the Sucrose/Amylase working mixture (Enzyme Solution 1) (see page 8, point 8). The released monosaccharides will then be removed in the alkaline borohydride step. If this treatment is included, the volume change will need to be considered in the calculations (i.e. the final volume of of **Solution S** (page 9), will be 1.15 mL instead of 1.1 mL.

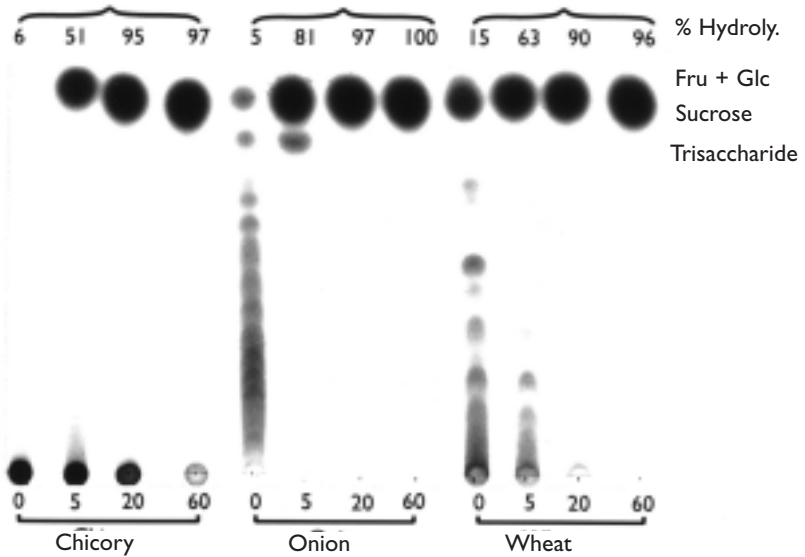


Figure 3. Thin layer chromatography of the sugars produced on hydrolysis of chicory, onion and wheat fructans by *exo*-inulinase. The reaction conditions are as described in the text. Samples were taken at 0, 5, 20 and 60 min for chromatography. TLC plates were developed once with *n*-propanol-ethanol-water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

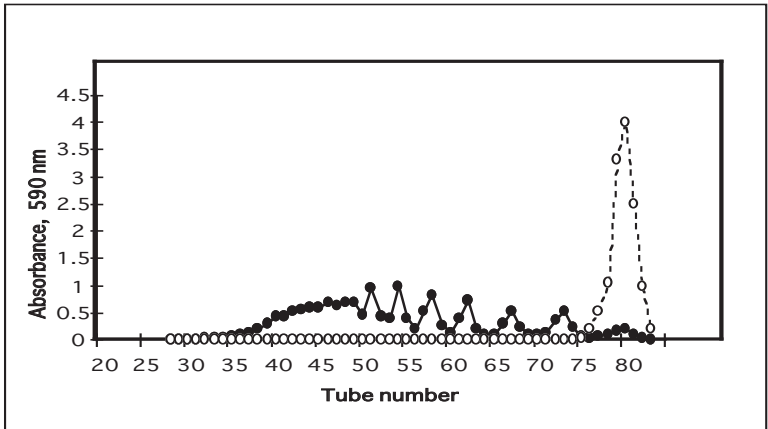


Figure 4. Bio-Gel P-2 chromatography of the sugars produced on hydrolysis of onion fructan by *exo*-inulinase. The reaction conditions are as described in the text. Column eluates were analysed by the phenol-sulphuric acid procedure. Incubated for 0 min (●) and 60 min (○) with *exo*-inulinase.

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** (100 U) plus β -amylase (*B. cereus*, 500 U), pullulanase (*K. pneumoniae*, 100 U) and maltase (yeast, 1,000 U) as a freeze-dried powder. Stable for > 5 years at -20°C .
- Bottle 2.** **Fructanase.** Highly purified *exo*-inulinase (10,000 U) and *endo*-inulinase (100 U) 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 (0.1 M, pH 6.5)] (Enzymes Solution 1). 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 the vial 2 in 22 mL of Buffer 2 [sodium acetate (0.1 M, pH 4.5)]. Divide into aliquots of appropriate volume and store at -20°C . Stable in polypropylene containers for > 5 years at -20°C .
- 3, 4 Use the contents of bottles 3, 4 and 5 as supplied.
- & 5. Stable for > 5 years at room temperature.

BUFFERS (not supplied):

- Buffer 1:** **Sodium maleate buffer** (0.1 M, 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** (0.1 M, 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 (0.2 M)

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 Multipipette®
 - with 5.0 mL Combitip® (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® (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. After the 6 min incubation time, the rack of tubes should be immediately immersed in a cold-water bath (at about 18-20°C) and left there for 5-10 min to cool. The absorbances of the reaction solutions should then be measured within 10 min.
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 0.1 M 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 [0.1 M 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** 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 % (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.a** (page 9) of the assay procedure. Treatment with fructanase enzyme (**Step C.ii**) 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. 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 **galactosyl-sucrose oligosaccharides**, these can be removed by incubation with *A. niger* α -galactosidase (Megazyme cat. no. E-AGLAN). Add 50 μ L 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 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:

- a. 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).
- b. Add 0.2 mL of diluted Sucrase/Amylase solution (**Enzyme A**) and incubate the tube at 40°C for 30 min.
- c. 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.
- d. Add 0.5 mL of **Reagent 4** (0.2 M 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:

- i. Accurately and carefully transfer 0.2 mL aliquots of **Solution S** into the bottom of three (3) glass test-tubes (16 x 100 mm).
- ii. Add 0.1 mL of fructanase solution (**Enzyme 2**) to two of these tubes (**samples**) and 0.1 mL of 0.1 M sodium acetate buffer to the third (**sample blank**).
- iii. Incubate the tubes at 40°C for 20 min to effect complete hydrolysis

of fructan to D-fructose and D-glucose.

- iv. 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.
- v. Remove the tubes from the boiling-water bath and immediately place them in cold water (18-20°C) for approx. 5 min.
- vi. 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.**

CALCULATIONS:

Fructan (% w/w as is):

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

where:

Δ_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).

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