

Megazyme

FRUCTAN HK PROCEDURE

for the measurement of

**FRUCTO-OLIGOSACCHARIDES
(FOS)**

and

FRUCTAN POLYSACCHARIDE

K-FRUCHK 03/05

A MODIFICATION OF

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 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. graminean 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 α -galactosidase, consequently, any galactosyl-sucrose oligosaccharides present in the sample will also be hydrolysed to fructose and glucose (and galactose). Separate from the possible problems with galactosyl-sucrose oligosaccharides, this method is quite complex, and requires the use of expensive equipment.

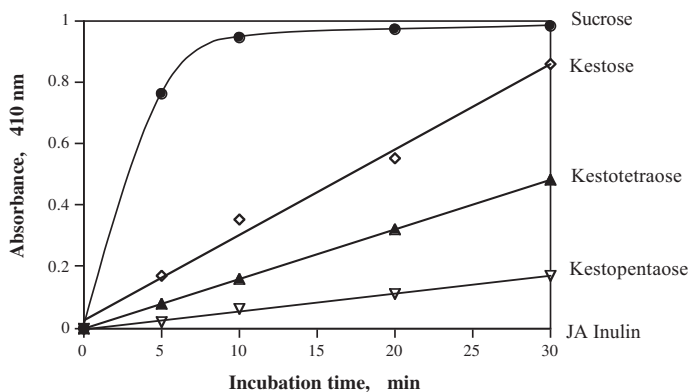


Figure 1. Hydrolysis of sucrose and fructans by yeast invertase.

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. 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^{1,2}) (**Figure 2**). At substrate concentrations of 10 mg/mL, the relative rate of hydrolysis of sucrose and 1-kestose is 3,800:1.

The method described here 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 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**).

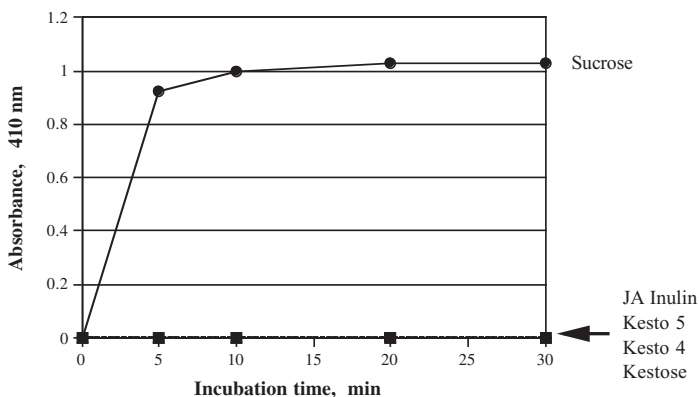


Figure 2. Hydrolysis of sucrose and fructans by sucrose.

Sugar compound (0.2 mL, 50 µg) 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.

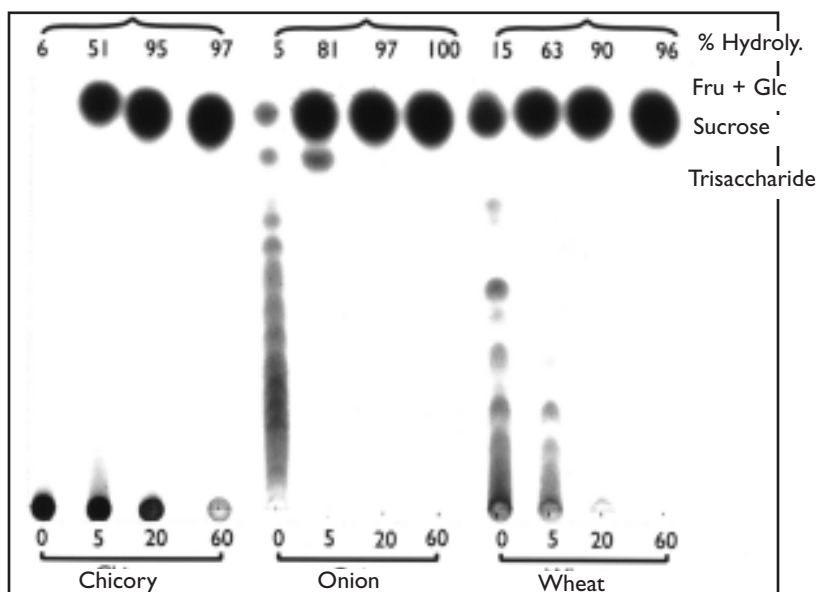


Figure 3. Thin layer chromatography of the sugars produced on hydrolysis of chicory, onion and wheat fructans by *exo*-inulinase (conditions as described in the text). Samples were taken at 0, 5, 20 and 60 min for chromatography. TLC plates were developed once with *n*-propanol-ethanol-water (7:1:2).

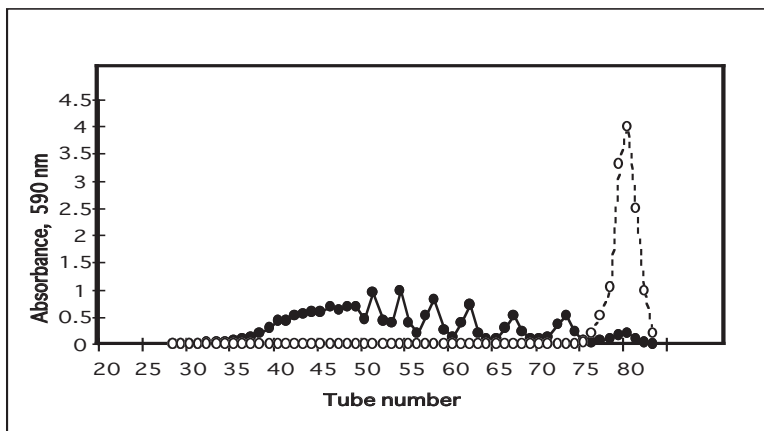


Figure 4. Bio-Gel P-2 chromatography of the sugars produced on hydrolysis of onion fructan by exo-inulinase. Incubation conditions as described in the text. Column eluates were analysed by the phenol-sulphuric

PRINCIPLE:

Sucrose and lower degree-of-polymerisation (DP) maltosaccharides (if present in the sample) are hydrolysed to fructose and glucose using a specific **sucrase/maltase** enzyme. After pH adjustment, samples are taken for analysis of glucose + fructose (A), or are treated with purified **fructanase** (which hydrolyses fructan to fructose and glucose) and then analysed for glucose + fructose (B). The concentration of glucose plus fructose is measured with a hexokinase/phospho-glucose isomerase/glucose 6-phosphate dehydrogenase system. Fructan content is then determined by the difference between B and A.

KITS:

Kits suitable for performing 50 determinations of fructan are available from Megazyme. The kits contain the full assay method plus:

- Bottle 1:** Imidazole buffer (25 mL, 2 M, pH 7.6) plus magnesium chloride (100 mM) and sodium azide (0.02 % w/v) as a preservative. Stable for > 2 years at 4°C.
- Bottle 2:** NADP⁺ (150 mg) plus ATP (440 mg). Stable for > 5 years at -20°C.
- Bottle 3:** Sucrase (100 U)/maltase (1,000 U), lyophilised powder plus BSA. Stable for > 5 years at -20°C.
- Bottle 4:** Fructanase (highly purified exo-inulinase (5,000 U) and endo-inulinanase (100 U), lyophilised powder. Stable for > 5 years at -20°C.

- Bottle 5:** Hexokinase (425 U/mL) plus glucose-6-phosphate dehydrogenase (212 U/mL) and PGI (840 U/mL) suspension, 2.25 mL. Stable for > 2 years at 4°C.
- Bottle 6:** D-Fructose standard solution (0.5 mg/mL) in 0.2 % (w/v) benzoic acid. Stable for > 2 years at room temperature.
- Bottle 7:** Fructan control flour. Dahlia fructan freeze-dried in the presence of α -cellulose. Stable for > 5 years stored dry at room temperature.

PREPARATION OF REAGENT SOLUTIONS/SUSPENSIONS:

1. Use the contents of bottle 1 as supplied. Stable for > 2 years at 4°C.
2. Dissolve the contents of bottle 2 in 12 mL of distilled water. Divide into aliquots of approx. 5 mL. Store on ice during use. Stable for > 2 years at -20°C.
3. Dissolve the entire contents of bottle 3 (sucrase/maltase) in 11 mL of **Buffer 1** (100 mM sodium maleate, pH 6.5; see below). Divide into aliquots of appropriate volume and store in polypropylene tubes between use. Stable for > 2 years at -20°C.
4. Dissolve the contents of bottle 4 (fructanase) in 11 mL of **Buffer 2** (100 mM sodium acetate, pH 4.5; see below). Divide into aliquots of appropriate volume and store in polypropylene tubes between use. Stable for > 2 years at -20°C.
5. Use the contents of bottle 5 as supplied. Before opening for the first time, shake the bottle to remove any protein that may have settled on the rubber stopper. Subsequently, store the bottle in an upright position. Stable for > 2 years at 4°C.
6. Use the contents of bottle 6 as supplied. Stable for > 2 years at room temperature.
7. Use as supplied. Stable for > 4 years at room temperature.

BUFFERS (not supplied):

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°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.

EQUIPMENT (RECOMMENDED):

1. Glass test tubes (round bottomed; 16 x 100 mm) and pyrex beakers (100 and 200 mL capacity).
2. Volumetric flasks (50 and 100 mL capacity).
3. Micro-pipettors, e.g. Gilson Pipetman® 200 µL, and 100 µL.
4. Positive displacement pipettor e.g. Eppendorf Multipipette®
- with 5.0 mL Combitip® (to dispense 0.2 mL aliquots of sucrase and imidazole buffer and 0.1 mL aliquots of fructanase).
5. Analytical balance.
6. Spectrophotometer set at 340 nm and disposable plastic cuvettes.
7. Vortex mixer (we recommend the IKA MS2 Minishaker®).
8. Thermostatted water bath (set at 40.0°C).
9. Dry hot-block heater (with holes adequate to accommodate 3.0 ml disposable plastic cuvettes). Optional.
10. Hot-plate magnetic stirrer.
11. Parafilm®
12. Bench centrifuge (capable of speeds of 1,000g) or filter funnels with Whatman No. 1 (9 cm) filter papers.
13. Stop clock.

CONTROLS AND PRECAUTIONS:

1. The time for complete conversion of fructose to 6-phospho gluconate is 10 min at 25°C. Ensure that this reaction is complete by checking the absorbance changes of the fructose standard in the reaction mixture.
2. Run a fructose standard solution with each set of determinations (to ensure that the HK/PGI/G6PDH reactions are progressing correctly) and analyse concurrently. In this assay, mix 0.1 mL of solution 6 (fructose standard; 0.5 mg/mL), 2.2 mL of distilled water, 0.2 mL of solution 1 (imidazole buffer) and 0.1 mL of solution 2 (NADP⁺/ATP solution) in a plastic cuvette. Measure the absorbance after 3 min (A_1). Add 0.02 mL of suspension 5 (HK/PGI/G6PDH), incubate for 10 min at 25°C and measure the absorbance at 340 nm (A_2).
3. Analyse the **fructan control powder** with each set of determinations. The fructan content of this powder is given on the vial label. Extract this as for samples containing 12-100 % w/w fructan (page 7), but reduce the sample weight and final volume 5-fold, i.e. extract 200 mg of sample in 80 ml of water at 80°C and then adjust the volume to 100 ml.

ASSAY PROCEDURE:

A. Fructan Extraction.

Mill dry samples to pass a 0.5 mm screen. Cut solid fatty samples (e.g. chocolate) into fine shavings with a sharp knife; analyse soft food products (e.g. spreads) without further preparation. Before they are weighed, all samples should be at room temperature.

Samples containing 0-12% fructan (plus sucrose control flour)

1. Accurately weigh 1.0 g of sample into a dry pyrex beaker (100 mL capacity) and add 40 mL of hot distilled water ($\sim 80^{\circ}\text{C}$). Place the beaker on a hot-plate, magnetic stirrer and stir and heat (at $\sim 80^{\circ}\text{C}$) 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 and adjust to volume with distilled water. Mix the contents thoroughly.
3. If the sample contains significant quantities of glucose, fructose and/or sucrose (say 30-60% w/w), aliquots will need to be diluted a further 5-fold or 10-fold before assay. Mix the solution thoroughly and repeat the assay.

Samples containing 12-100% fructan (or fructan plus sugars)

1. Accurately weigh approximately 1.0 g of sample into a dry pyrex beaker (800 mL capacity) and add 400 mL of hot distilled water ($\sim 80^{\circ}\text{C}$). Place the beaker on a hot-plate, magnetic-stirrer and stir and heat (at $\sim 80^{\circ}\text{C}$) 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 500 mL volumetric flask and adjust the volume to the mark with distilled water. Mix the contents thoroughly.

B. Further treatment of samples.

1. Filter an aliquot of the solution through a Whatman No. 1 (9 cm) filter circle and analyse it immediately. If the solution is still turbid, filter an aliquot through Whatman GF/A glass fibre filter paper.

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 $\sim 80^{\circ}\text{C}$ and allowed to cool to room temperature before samples are removed for analysis.

C. Hydrolysis of Sucrose and low DP Maltosaccharides.

1. Accurately dispense 0.2 mL aliquots of solutions to be analysed (containing approximately 0.1 to 2.0 mg/mL of fructan) into the bottom of glass test-tubes (16 x 100 mm).
2. Add 0.2 mL of solution 3 (sucrase/maltase mixture) to each tube and incubate the tubes at 40°C for 30 min.
3. Add 0.5 mL of buffer 2 (100 mM sodium acetate buffer, pH 4.5) to each tube with vigorous stirring on a vortex mixer. This is termed **Solution A**.

D. Hydrolysis of Fructan.

1. Accurately and carefully dispensed 0.2 mL aliquots of **Solution A** (in duplicate) to the bottom of plastic spectrophotometer cuvettes (3 ml volume, 1 cm lightpath).
2. Add 0.1 mL of solution 4 (fructanase solution) to the bottom of one cuvette, and 0.1 mL of buffer 2 (100 mM sodium acetate buffer, pH 4.5) to the second cuvette. Mix the contents thoroughly and cover the cuvette with Parafilm®.
3. Incubated the covered cuvettes at 40°C for 20 min in a dry hot-block heater to effect complete hydrolysis of fructan to fructose and glucose (in the cuvettes containing the fructanase enzyme).

E. Measurement of fructose and glucose.

PROCEDURE:

Wavelength:	340 nm
Cuvette:	1 cm light path (glass or plastic)
Temperature:	~ 25°C
Final volume:	2.62 mL
Sample solution:	4-100 µg of D-glucose and D-fructose per cuvette (in 0.30 mL sample volume).

Read against air (without a cuvette in the light path) or against water.

Contents of cuvettes	Blank	Reaction
Distilled water	2.00 mL	2.00 mL
sample + buffer 2 (acetate buffer)	0.30 mL	-
sample + solution 4 (fructanase)	-	0.30 mL
solution 1 (imidazole buffer)	0.20 mL	0.20 mL
solution 2 (NADP ⁺ /ATP)	0.10 mL	0.10 mL
mix*, read the absorbances of the solutions (A_1) after approximately 3 min and start reaction by addition of		
suspension 5 (HK/PGI/G-6-PDH)	0.02 mL	0.02 mL
mix*, wait for the end of the reaction (approx 10-15 min), and read the absorbances of the solutions (A_2). If the reaction has not stopped after 15 min, continue to read the absorbances at 5-min intervals until the absorbances remain the same over 5 min**.		

* for example with a plastic spatula or by gentle swirling after closing the cuvette with Parafilm®.

** If the colour continues to increase, this may be due to effects of colour compounds or enzymes in the sample. These interfering substances may be removed during sample preparation.

Determine the absorbance difference ($A_2 - A_1$) for both blank and reaction (fructanase treated). Subtract the absorbance difference of the blank from the absorbance difference of the reaction, thereby obtaining $\Delta A_{\text{fructan}}$.

CALCULATIONS:

Fructan (% w/w as is):

$$= \Delta A_{\text{fructan}} \times F \times 5 \times \frac{0.9}{0.2} \times V \times \frac{100}{W} \times \frac{1}{1000} \times \frac{162}{180}$$
$$= \Delta A_{\text{fructan}} \times F/W \times V \times 2.025$$

where:

$$\Delta A_{\text{fructan}} = [\Delta(A_2 - A_1)_{\text{reaction}}] - [\Delta(A_2 - A_1)_{\text{blank}}]$$

F = factor to convert absorbance values to μg fructose.
= (50 μg fructose)/(absorbance value for 50 μg fructose).

5 = Factor to convert from 0.2 mL as assayed to 1.0 mL.

$\frac{0.9}{0.2}$ = 0.2 mL was taken from 0.9 mL of enzyme digest for analysis.

V = volume (mL) of extractant used (i.e. 100 or 500 mL).

W = weight (mg) of sample extracted.

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

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

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

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

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**Megazyme International Ireland Ltd.,
Bray Business Park, Bray,
Co. Wicklow,
IRELAND**

Telephone: (353.1) 286 1220

Facsimile: (353.1) 286 1264

Internet: www.megazyme.com

E-Mail: info@megazyme.com

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