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FRUCTAN HK

ASSAY PROCEDURE

for the measurement of

FRUCTO-OLIGOSACCHARIDES (FOS)

and

FRUCTAN POLYSACCHARIDE

K-FRUCHK 03/20

(with recombinant inulinases)

(50 Assays per Kit)

A modification of:
AOAC Method 999.03
AACC Method 32.32.01

Not suitable for analysis of samples with high levels of D-glucose, D-fructose, sucrose or maltose.
- Refer to K-FRUC -

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INTRODUCTION:

Fructans are defined as any compound where one or more fructosyl-fructose linkage constitutes a majority of the linkages.[1,2] 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.[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 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. [3,4] This introduces the problem of independently removing, or measuring, sucrose, D-fructose and D-glucose. Pontis (1966)[4,5] has reported the removal of sucrose, D-glucose and D-fructose by hydrolysing sucrose with a crystalline yeast invertase. However, yeast invertase also hydrolyses lower degree of polymerisation (DP) fructo-oligosaccharides (FOS).[3]

An alternative approach[6] 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 currently described method[3,7-9] 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 onion and agave (*Agavaceae*)[3,7-9] is the most resistant, but even this is completely hydrolysed under the described assay conditions. This method is not applicable to the measurement of levan. Because this method does not include a borohydride reduction step (as used in **K-FRUC**), partially hydrolysed inulins (e.g. Raftilose P-95®) are not underestimated. However, the method is not suitable for use on samples that contain high levels of D-glucose, D-fructose, sucrose or maltose as these contribute to the sample blank absorbance. This will lead to large errors if the fructan content is low.

PRINCIPLE:

Sucrose and lower degree-of-polymerisation (DP) maltosaccharides (if present in the sample) are hydrolysed to D-fructose and D-glucose using a specific sucrase/maltase enzyme (McCleary and Blakeney, 1999). Starch and higher DP maltodextrins are not hydrolysed. (1) (2).

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

$$\text{(2) Maltose} + \text{maltotriose} \xrightarrow{\text{(maltase, pH 6.5, 40°C)}} \text{D-glucose}$$

After pH adjustment, the blank values of samples are determined by direct analysis of D-glucose plus D-fructose using the hexokinase/ phosphoglucose isomerase/glucose 6-phosphate dehydrogenase analytical procedure (3) (4).

$$\text{(3) D-Glucose} + \text{D-fructose} + \text{ATP} \xrightarrow{\text{HK + PGI}} \text{G-6-P} + \text{ADP}$$

$$\text{(4) G-6-P} + NADP^+ \xrightarrow{\text{(G6P-DH)}} \text{gluconate-6-phosphate} + \text{NADPH} + H^+$$

The amount of NADPH formed in this reaction is stoichiometric with the amount of D-glucose plus D-fructose. It is the NADPH which is measured by the increase in absorbance at 340 nm.

The fructan content of samples is determined by incubating aliquots of the same solution with ultra-pure (recombinant/affinity purified) *exo*- and *endo*-inulinases (to hydrolyse fructan to D-fructose and D-glucose) (5). These solutions and are then analysed for D-glucose plus D-fructose (3)(4). Fructan content is determined by subtracting absorbance values from sample blank from those for the sample.

$$\text{(5) Fructan} + H_2O \xrightarrow{\text{\textit{exo}- and \textit{endo}-inulinases, pH 4.5, 40°C}} \text{D-glucose} + \text{D-fructose}$$

SPECIFICITY, SENSITIVITY, LINEARITY AND PRECISION:

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). The hexokinase/phosphoglucose isomerase/glucose 6-phosphate dehydrogenase assay procedure is specific for D-glucose and D-fructose.

The smallest differentiating absorbance for the assay is 0.010 absorbance units. This corresponds to 33.7 mg/L of sample solution (before incubation with sucrase/maltase and dilution with acetate buffer) at a sample volume of 0.20 mL. The detection limit is 67.4 mg/L, which is derived from an absorbance difference of 0.020 with a sample volume of 0.20 mL.

The assay is linear over the range of 4 to 80 µg of D-glucose or D-fructose per assay. In duplicate determinations using one sample solution, an absorbance difference of 0.005 to 0.010 may occur. With a sample volume of 0.20 mL, this corresponds to a D-glucose/D-fructose concentration of approx. 16.9 to 33.7 mg/L of sample solution. If the sample is diluted during sample preparation, the result is multiplied by the dilution factor, F. If, in sample preparation, the sample is weighed, e.g. 10 g/L, a difference of 0.02 to 0.05 g/100 g can be expected.

This method is based on the **K-FRUC** assay procedure for fructan. That procedure was the subject of a successful interlaboratory evaluation under the auspices of AOAC International and AACC International.[7,9] Sucrose, maltose, maltodextrins and starch are not measured. Galactosyl-sucrose oligosaccharides, if present, must be enzymatically removed. See Controls and Precautions 4, page 5.

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.

KITS:

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

Bottle 1: Buffer (25 mL, pH 7.6) plus sodium azide (0.02% w/v) as a preservative.
Stable for > 2 years at 4°C.

Bottle 2: $NADP^+$ plus ATP.
Stable for > 5 years below -10°C.

Bottle 3: Sucrase/maltase, lyophilised powder plus BSA.
Stable for > 5 years abelow -10°C.

Bottle 4: Fructanase (recombinant, affinity purified *exo*-inulinase and *endo*-inulinase), lyophilised powder.
Stable for > 5 years below -10°C.

Bottle 5: Hexokinase plus glucose-6-phosphate dehydrogenase and PGI suspension, 2.25 mL.
Stable for > 4 years at 4°C.

Bottle 6: D-Fructose standard solution (0.5 mg/mL) in 0.2% (w/v) benzoic acid.
Stable for > 4 years; store sealed at room temperature.

Bottle 7: Fructan control flour. Dahlia fructan freeze-dried in the presence of α-cellulose.
Stable for > 5 years; store sealed 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. **Stable for > 1 year at 4°C** or stable for > 2 years below -10°C (to avoid repetitive freeze/thaw cycles, divide into appropriately sized aliquots and store in polypropylene tubes).

3. Dissolve the contents of bottle 3 (sucrase/maltase) in 11 mL of Buffer 1 **(B)** (100 mM sodium maleate, pH 6.5 plus BSA). Divide into aliquots of appropriate volume and store in polypropylene tubes between use.
Stable for > 2 years below -10°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 below -10°C.

5. Use the contents of bottle 5 as supplied. Before opening for the first time, swirl the bottle to remove any protein that

may have settled on the rubber stopper. Subsequently, store the bottle in an upright position.
Stable for > 4 years at 4°C.

6. Use the contents of bottle 6 as supplied.
Stable for > 4 years; store sealed at room temperature.

7. Use the contents of bottle 7 as supplied.
Stable for > 5 years; store sealed at room temperature.

BUFFERS (NOT SUPPLIED):

1A. Sodium maleate buffer (100 mM, pH 6.5).
Dissolve maleic acid (11.6 g, Sigma cat. no. M0375) 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.

1B. To 100 mL of Buffer 1 **(A)** add 50 mg of Bovine serum albumin and 0.02 g of sodium azide. Use this buffer to dissolve the contents of bottle 3 (sucrase preparation). Store buffer 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 L. Store at 4°C.

EQUIPMENT (RECOMMENDED):

1. Pyrex screw cap culture tubes (25 x 150 mm) with PTFE lined phenolic caps (Fisher cat. no. 14-933D).
2. Volumetric flasks (50 and 100 mL capacity).
3. Micro-pipettors, e.g. Gilson Pipetman® (100 µL and 200 µL).
4. Positive displacement pipettor, e.g. Eppendorf Multipette®
   - 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. Thermostated water baths set at 30°C and 40°C.
9. Dry hot-block heater (with holes adequate to accommodate 3.0 mL disposable plastic cuvettes). Optional.
10. Microfuge (required speed 13,000 rpm) and disposable 2.0 mL polypropylene microfuge tubes, e.g. Sarstedt cat. no. 72.691.

CONTROLS AND PRECAUTIONS:

1. The time for complete conversion of fructose to 6-phosphogluconate 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.
4. 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 (after Step C.1) and incubate for 30 min at 40°C before addition of the Sucrase/Maltase working mixture (Enzymes Solution 1). This enzyme gives complete hydrolysis of D-galactose from galactosyl-sucrose oligosaccharides.

ASSAY PROCEDURE:

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.

A. Fructan Extraction

Samples containing 0-12% fructan

1. Accurately weigh 1.0 g of sample into a Pyrex screw cap culture tubes (25 x 150 mm) and add 25 mL of distilled water. Loosely cap the tube. Place the tube into a boiling water bath and heat for 5 min, tighten the tube cap and vigorously mix the contents. Heat the tube contents for a further 5 min and mix the contents.
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 D-glucose,

D-fructose and/or sucrose (say 30-60% w/w), aliquots should be diluted a further 5-fold or 10-fold before assay. Mix the solution thoroughly and repeat the assay. For samples containing high levels of D-glucose, D-fructose, sucrose and/or maltose, the **K-FRUC** assay kit and protocol should by followed.

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

1. Accurately weigh approx. 1.0 g of sample into a dry pyrex flask (500 mL capacity) and add 300 mL of hot distilled water. Place the flask in a boiling watrer bath and heat (at ~ 100°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. Transfer ~ 2.0 mL of samples solutions into 2.0 mL microfuge tubes and centrifuge at 13,000 rpm for 5 min. Alternatively, filter an aliquot of the solution through a Whatman GF/A glass fibre filter paper. Analyse the sample soon after centrifugation or filtering. 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 centrifugation and analysis.

C. Hydrolysis of Sucrose and low DP Maltosaccharides

1. Accurately dispense 0.2 mL aliquots of solutions to be analysed (containing approx. 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 30°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 dispense 0.2 mL aliquots of **Solution A** (in duplicate) to the bottom of plastic spectrophotometer cuvettes (3 mL volume, 1 cm light path).
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. Incubate the covered cuvettes at 40°C for 30 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 fructan

PROCEDURE:

Wavelength: 340 nm
Cuvette: 1 cm light path (glass or plastic)
Temperature: ~ 40°C and 25°C
Final Volume: 2.62 mL
Sample Solution: 4-100 µg of D-glucose and D-fructose per cuvette (in 0.20 mL sample volume)

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

Pipette into cuvettes	Sugars	Fructan + sugars
sample	0.20 mL	0.20 mL
solution 4 (fructanase enzymes)	-	0.10 mL
buffer 2 (sodium acetate buffer)	0.10 mL	-
Ensure that all of the solutions are delivered to the bottom of the cuvette. Mix the contents by gentle swirling, cap the cuvettes and incubate them for 30 min at 40°C in a heated hot-block or oven. **Add:**		
distilled water (at ~ 25°C)	2.00 mL	2.00 mL
solution 1 (buffer, pH 7.6)	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 approx. 3 min and start the reactions by addition of:		
suspension 5 (HK/PGI/G-6-PDH)	0.02 mL	0.02 mL
Mix*. Incubate the cuvettes at ~ 25°C for 15 min. At this time, the absorbances will remain constant.** Record the absorbance (A_2).		

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

** If the colour continues to increase after 15 min, this may be due to effects of colour compounds in the sample. These interfering substances may be removed during sample preparation.

CALCULATION:

Determine the absorbance difference (A_2-A_1) for both "*sugars*" and "*fructan + sugars*" and calculate values for ΔA_{sugars} and $\Delta A_{fructan\ +\ sugars}$ as described below.

Determination of D-fructose + D-glucose in the "sugars" sample:

ΔA_{sugars} = (A_2-A_1) (from the "*sugars*" sample).

Determination of D-fructose + D-glucose in the "fructan + sugars" sample:

$\Delta A_{fructan\ +\ sugars}$ = (A_2-A_1) (from the "*fructan + sugars*" sample).

The values of ΔA_{sugars} and $\Delta A_{fructan\ +\ sugars}$ should as a rule be at least 0.100 absorbance units to achieve sufficiently accurate results.

The concentration of "*sugars*" and "*fructan + sugars*" can be calculated as follows:

$$c = \frac{V \times MW}{\varepsilon \times d \times v} \times \frac{0.9}{0.2} \times \Delta A \quad [g/L]$$

where:

V = final volume [mL]

MW = molecular weight of D-glucose or D-fructose [g/mol]

ε = extinction coefficient of NADPH at 340 nm
= 6300 [l x mol^{-1} x cm^{-1}]

d = light path [cm]

v = sample volume [mL]

0.9/0.2 = 0.2 mL of sample was incubated with 0.2 mL sucrase/maltase enzyme and 0.5 mL acetate buffer added (total 0.9 mL); 0.2 mL of this was taken for incubation with fructanase enzymes (i.e. 0.2 mL removed from 0.9 mL).

It follows for "sugars":

$$c = \frac{2.62 \times 180.16}{6300 \times 1.0 \times 0.2} \times \frac{0.9}{0.2} \times \Delta A_{sugars} \quad [g/L]$$

$$= 1.6858 \times \Delta A_{sugars} \quad [g/L]$$

for "fructan + sugars":

$$c = \frac{2.62 \times 180.16}{6300 \times 1.0 \times 0.2} \times \frac{0.9}{0.2} \times \Delta A_{fructan\ +\ sugars} \quad [g/L]$$

$$= 1.6858 \times \Delta A_{fructan\ +\ sugars} \quad [g/L]$$

for "fructan":

$$c_{(fructan)} = c_{(fructan + sugars)} - c_{(sugars)} \times \frac{162}{180} \quad [g/L]$$

162/180 = factor to convert from free fructose and glucose as determined, to anhydrofructose and anhydroglucose as occurs in fructan.

If the sample has been diluted during preparation, the result must be multiplied by the dilution factor.

When analysing solid and semi-solid samples which are weighed out for sample preparation, the content (g/100 g) is calculated from the amount weighed as follows:

Content of "fructan"

$$= \frac{c_{fructan}\ [g/L\ sample\ solution]}{weight_{sample}\ [g/L\ sample\ solution]} \times 100 \quad [g/100\ g]$$

where 1 g sample is extracted in 50 mL:

"$weight_{sample}$ [g/L sample solution]" = 20

where 1.0 g sample is extracted in 500 mL:

"$weight_{sample}$ [g/L sample solution]" = 2

NOTE: Calculations can be simplified by using the Megazyme ***Mega-Calc***™, downloadable from where the product appears on the Megazyme website (www.megazyme.com).

REFERENCES:

1. Lewis, D. H. (1993). Nomenclature and diagrammatic representation of oligomeric fructans - a paper for discussion. *New Phytologist,* **124,** 583-594.
2. Uchiyama, T. (1993). Metabolism in microorganisms. Part II. Biosynthesis and degradation of fructans by microbial enzymes other than levansucrase. *"Science and technology of fructans"* (M. Suzuki and N. J. Chatterton, Eds.), CRC Press, pp 170-190.
3. McCleary, B. V. & Blakeney, A. B. (1999). Measurement of inulin and oligofructan. *Cereal Foods World*, **44**, 398-406.
4. Pontis, H. G. (1966). Observations on the *de novo* synthesis of fructosans *in vivo*. *Arch. Biochem. Biophys.*, **116**, 416-423.
5. Pontis, H. G. (1990). Fructans. *"Methods in Plant Biochemistry"* (P. M. Dey and J. B. Harborne, Eds.), Academic Press, New York, pp 353-369.

6. Quemener, B., Thibault, J. -F. & Coussement, P. (1993). Determination of inulin and oligofructose in food products, and integration in the AOAC method for measurement of total dietary fibre. *Lebansm.-Wiss. u. Technol.,* **27**, 125-132.6.

7. McCleary, B. V, Murphy, A. & Mugford, D. C. (2000). Determination of Oligofructans and Fructan Polysaccharides in Foodstuffs by an Enzymatic/ Spectrophotometric Method: Collaborative Study. *J. AOAC International,* **83**, 356-364.

8. McCleary, B. V. & Rossiter, P. (2004). Measurement of novel dietary fibres. *J. AOAC International*, **87**, 707-711.

9. McCleary B.V., Charmier, L. M. J., McKie, V. A., McLoughlin, C. & Rogowski, A. (2019). Determination of fructan (inulin, FOS, levan, and branched fructan) in animal foods (animal feed, pet foods and ingredients): Single-laboratory validation, First Action 2018.07. *J. AOAC International,* **102**, 883-892.

Megazyme

Bray Business Park, Bray,
Co. Wicklow,
A98 YV29,
IRELAND.

Telephone: (353.1) 286 1220
Facsimile: (353.1) 286 1264
Internet: www.megazyme.com
E-Mail: info@megazyme.com