FOOD COMPOSITION AND ADDITIVES

Measurement of Total Fructan in Foods by Enzymatic/ Spectrophotometric Method: Collaborative Study

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An AOAC collaborative study was conducted to evaluate the accuracy and reliability of an enzyme assay kit procedure for measuring oligofructans and fructan polysaccharide (inulins) in mixed materials and food products. The sample is extracted with hot water, and an aliquot is treated with a mixture of sucrase (a specific sucrose-degrading enzyme), α-amylase, pullulanase, and maltase to hydrolyze sucrose to glucose and fructose, and starch to glucose. These reducing sugars are then reduced to sugar alcohols by treatment with alkaline borohydride solution. The solution is neutralized, and excess borohydride is removed with dilute acetic acid. The fructan is hydrolyzed to fructose and glucose using a mixture of purified *exo*- and *endo*-inulinanases (fructanase mixture). The reducing sugars produced (fructose and glucose) are measured with a spectrophotometer after reaction with *para*-hydroxybenzoic acid hydrazide. The samples analyzed included pure fructan, chocolate, low-fat spread, milk powder, vitamin tablets, onion powder, Jerusalem artichoke flour, wheat stalks, and a sucrose/cellulose control flour. Repeatability relative standard deviations ranged from 2.3 to 7.3%; reproducibility relative standard deviations ranged from 5.0 to 10.8%.

Fructans are defined as any compound in which one or more fructosyl–fructose linkages constitute a majority of the linkages (1). This refers to polymeric materials 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 that can be specifically characterized and those that cannot (1).

In recent years, interest in measurement of fructans such as inulin and oligofructose has been stimulated by applications made to regulatory authorities that fructans be incorporated into dietary fiber for food labeling purposes. Like soluble dietary fiber, fructans are not metabolized in the human upper digestive tract but are fermented in the large bowel. Because fructans are largely soluble in 80% ethanol, they are not significantly measured by present dietary fiber methods. However, if fructans can be separately measured, accurately and precisely, they could be added to total soluble dietary fiber after fructans measured in dietary fiber residues are deducted.

Several procedures have been described for measuring fructan in plant material and food products, which are best measured after hydrolysis to fructose (and glucose). This introduces the problem of independently removing, or measuring, sucrose, fructose and glucose. Pontis (2) removed sucrose, glucose, and fructose by hydrolyzing sucrose with a crystalline yeast invertase and destroying the resulting glucose, fructose, and 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 incubation conditions. In testing currently available pure yeast invertases, we have found it extremely difficult, if not impossible, to achieve these conditions. We found that 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 approximately 100, 20, 10, 3, and 0%, respectively (3).

In an alternative approach (4, 5), capillary gas chromatography (CGC) or liquid chromatography (LC) is used to analyze extracts of samples either non-treated, or treated with amyloglucosidase or amyloglucosidase plus inulinase

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(fructanase). By measuring sucrose, fructose, and glucose in the various samples, and with appropriate calculations, free glucose and fructose, sucrose, starch, and fructan can be estimated (5). The possible interference of galactosyl–sucrose 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 high levels of β-glucanase; 6). Consequently, galactosyl–sucrose oligosaccharides present in the sample are also hydrolyzed to fructose and glucose (and galactose), which are measured as fructan. Aside from the possible problems with galactosyl–sucrose oligosaccharides, this method is quite complex, and requires the use of expensive equipment.

The presence of an active β-glucanase in the crude fructanase preparation precludes the use of this enzyme preparation in integrated inulin/AOAC dietary fiber methods (where samples contain β-glucan) as proposed by Quemener et al. (7). This problem can be resolved by using preparations in which the β-glucanase has been removed or reduced to an acceptable level (6).

In contrast, the method evaluated in the current study is accurate, reproducible, and specific; it is easy to perform, and uses standard laboratory equipment and highly purified and specific enzymes to hydrolyze sucrose, starch, and fructans. The sucrase enzyme rapidly hydrolyzes sucrose but has no activity on 1-kestose and other fructose oligosaccharides (8). At substrate concentrations of 10 mg/mL, the rate of hydrolysis of kestose and other fructans is <0.01% that of sucrose. The method can be used to measure fructan in plant materials and food mixtures.

Collaborative Study

Ten (coded) homogeneous test samples (containing fructan, or to which fructan was added as a supplement) of chocolate (A, M), milk powder (B, F), vitamin tablets (C, E), wheat stalks (I, L), sucrose/cellulose (K, Q), low-fat spread (S, T), onion powder (D, O), fructan control (G, R), pure fructan (H, P), and Jerusalem artichoke (J, N) were provided as 20 blind duplicates to 15 collaborators. The sucrose/cellulose material was included as a control sample. A reference sample (fructan control flour) was also supplied to familiarize collaborators with the method. To facilitate selection of test sample weights, laboratories were advised that the first 6 coded materials ranged from 0 to 12 % total fructan content, and the remainder ranged from 12 to 50 % fructan. Collaborators were requested to perform single determinations on each material by the enclosed method, but duplicate assays on aliquots of test material extract were also requested. Results were evaluated according to AOAC guidelines (9). Outlier results identified by the Cochran test for extremes of repeatability and the Grubbs test for extremes of reproducibility were omitted from further calculations. Also determined were within (s_r) and between (s_R) laboratory standard deviations, repeatability (r) and reproducibility (R) as $2.8 \times s_r$ and $2.8 \times s_R$, respectively, and relative standard deviations (RSD_r) and (RSD_R) from s_r and s_R as percentages of mean values.

999.03 Measurement of Total Fructan in Foods, Enzymatic/Spectrophotometric Method

First Action 1999

Method validated for the measurement of fructan in selected foods.

Method Performance

See Tables **999.03A** and **999.03B** for method performance data.

A. Principle

Samples are extracted with hot water to dissolve fructan. Aliquots of extract are treated with a specific sucrase to hydrolyze sucrose to glucose and fructose, and with a mixture of pure starch-degrading enzymes to hydrolyze starch to glucose. All reducing sugars are then reduced to the sugar alcohols by treatment with alkaline borohydride. The solution is neutralized and excess borohydride is removed with dilute acetic acid. The fructan is then hydrolyzed to fructose and glucose with purified fructanase (*exo*-inulinase plus *endo*-inulinase), and these sugars are measured with the *para*-hydroxybenzoic acid hydrazide (PAHBAH) method for reducing-sugars.

B. Apparatus

(**a**) *Grinding mill*.—Centrifugal, equipped with 12-tooth rotor and 0.5 mm sieve, or similar device. Alternatively, cyclone mill may be used for small samples.

(**b**) *Hot plate.*—With magnetic stirrer.

(**c**) *Water bath.*—Capable of maintaining 40° ± 0.1°C.

(**d**) *Boiling water bath.*—Capable of boiling (e.g., appropriate deep-fat fryer filled with water) at 95°–100°C.

(**e**) *Vortex mixer.*

(**f**) *pH Meter.*

(**g**) *Stop-clock timer.*

(**h**) *Filter paper.*—Fast, e.g., Whatman No. 1 or equivalent.

(**i**) *Vacuum oven.*—For drying fructose standard.

(**j**) *Spectrophotometer*.—Capable of operating at 410 nm.

(**k**) *Pipets.*—Capable of delivering 100 and 200 µL; with disposable tips. Alternatively, motorized hand-held dispenser may be used.

(**l**) *Positive displacement pipetter.*—Equipped with tips capable of accurately delivering 100 and 200 µL, and tips capable of delivering 5.0 mL.

(**m**) *Glass test tubes.*—16 × 120 mm, round bottom, 17 mL capacity.

(**n**) *Volumetric flasks.*—50 and 100 mL capacity.

(**o**) *Polypropylene containers*.—10 mL capacity with screw cap.

C. Reagents

All reagents should be of analytical purity grade.

(**a**) *Sodium maleate buffer.*—100 mM, pH 6.5. Dissolve 11.6 g maleic acid in 900 mL distilled water and adjust pH to

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Table 999.03A. Results for determination of total fructan in food products and plant materials as g/100 g material analyzed

Sample	Chocolate	Milk powder	Vitamin tablet	Onion	Fructan control
No. labs[a]	13	13	13	10	13
No. outliers[b]	0	0	0	3	0
Mean	13.52	10.64	4.60	51.43	28.47
s_r[c]	0.87	0.72	0.33	2.41	1.89
s_R[d]	1.34	1.10	0.46	3.18	2.48
RSD_r[e]	6.4	6.7	7.1	4.7	6.6
RSD_R[f]	9.9	10.4	9.9	6.2	8.7
r[g]	2.4	2.0	0.9	6.8	5.3
R[h]	3.7	3.1	1.3	8.9	7.0

[a] No. labs = number of laboratories included in calculations.
[b] No. outliers = number of outlier laboratories, not included in calculations.
[c] s_r = repeatability standard deviation.
[d] s_R= reproducibility standard deviation.
[e] RSD_r = repeatability relative standard deviation.
[f] RSD_R = reproducibility relative standard deviation.
[g] r = repeatability value ($2.8 \times s_r$).
[h] R = reproducibility value ($2.8 \times s_R$).

6.5 with 2M NaOH solution; adjust volume to 1 L in volumetric flask. Store buffer at 4°C.

(**b**) *Sodium acetate buffer.*—100 mM, pH 4.5. Pipet 5.8 mL glacial acetic acid (1.05 g/mL) to 900 mL distilled water. Adjust to pH 4.5 using 1M NaOH. Dilute to 1 L with water. Store at 4°C.

(**c**) *PAHBAH reducing sugar assay reagent*.—(*1*) *Solution A.*—Add 10 g PAHBAH (e.g., Sigma Cat. No. H-9882, Sigma Chemical Co., St. Louis, MO) to 60 mL water in 250 mL beaker on magnetic stirrer. Stir slurry and add 10 mL concentrated HCl. Adjust to 200 mL with distilled water and store at room temperature (ca 22°C). Stable for at least 2 years. (*2*) *Solution B.*—Add 24.9 g trisodium citrate to 500 mL distilled water and stir to dissolve. Add 2.20 g calcium chloride dihydrate and dissolve by stirring. Then add 40.0 g NaOH and dissolve with stirring. (Solution may be milky, but will clarify on dilution). Adjust volume to 2 L. The solution is stable for at least 2 years at room temperature (ca 22°C). (*3*) *PAHBAH working reagent.*—Immediately before use, add 20 mL Solution A to 180 mL Solution B and mix thoroughly. This solution should be stored on ice and is stable for ca 4 h.

(**d**) *Sodium hydroxide (50 mM).*—Dissolve 2.0 g NaOH in 900 mL distilled water. Adjust volume to 1 L. Store at room temperature (ca 22°C).

(**e**) *Alkaline borohydride (10 mg/mL sodium borohydride in 50 mM NaOH).*—Accurately weigh 50 mg sodium borohydride (Sigma Cat. No. S-9125) into polypropylene containers (10 mL volume with screw cap). Record weight on tubes (ca 10 for convenience); seal tubes and store them in a desiccator for future use. Immediately before use, dissolve sodium borohydride (at 10 mg/mL) in 50 mM NaOH solution [reagent *C*(**d**)]. This solution is stable for 4–5 h at room temperature.

(**f**) *Acetic acid (100 mM).*—Add 5.8 mL glacial acetic acid to distilled water and adjust volume to 1 L. Store at room temperature (ca 22°C).

(**g**) *Sucrase/amylase preparation.*—2.27 units (U) sucrase/mL. Dissolve contents of 1 vial containing sucrase (50 U) plus β-amylase (*Bacillus cereus*, 500 U), pullulanase (*Bacillus licheniformis,* 100 U) and maltase (yeast, 1000 U; as freeze-dried powder) in 22 mL sodium maleate buffer *C*(**a**). Divide enzyme solution into 5 mL aliquots, and store frozen in polypropylene containers to prevent microbial contamination. If not diluted in buffer, the freeze-dried enzyme is stable 5 years when stored at –20°C. One unit sucrase activity is the amount of enzyme required to release 1 μmole glucose/min from sucrose at pH 6.5 and 40°C.

(**h**) *Fructanase solution*.—350 U/mL *exo*-inulinase and 35 U/mL *endo*-inulinase. Dissolve contents of 1 vial containing 8000 U *exo*-inulinase and 800 U *endo*-inulinase in 22 mL sodium acetate buffer, *C*(**b**). Divide enzyme solution into 5 mL aliquots, and store frozen in polypropylene containers to prevent microbial contamination. If not diluted in buffer, the freeze-dried enzyme is stable 5 years when stored at –20°C. One unit (U) *exo*-inulinase activity is the amount of enzyme required to release 1 μmole of reducing-sugar equivalents (as fructose)/min from kestose (10 mg/mL) at pH 4.5 and 40°C.

(**i**) *Fructan control flour.*—Containing a known amount of dahlia fructan freeze-dried in the presence of α-cellulose. Stable when stored dry at room temperature.

(**j**) *Sucrose control flour.*—Sucrose freeze-dried in the presence of α-cellulose. Stable when stored dry at room temperature (ca 22°C).

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Table 999.03B. Results for determination of total fructan in food products and plant materials as g/100 g material analyzed

Sample	Pure fructan	Wheat stalks	Jerusalem artichoke	Low fat spread
No. labs[a]	10	13	11	13
No. outliers[b]	2	0	2	0
Mean	95.87	4.72	51.63	8.19
s_r[c]	2.65	0.11	2.44	0.59
s_R[d]	4.75	0.41	2.95	0.88
RSD_r[e]	2.8	2.3	4.7	7.3
RSD_R[f]	5.0	8.6	5.7	10.8
r[g]	7.4	0.3	6.8	1.7
R[h]	13.3	1.1	8.3	2.5

[a] No. labs = number of laboratories included in calculations.
[b] No. outliers = number of outlier laboratories, not included in calculations.
[c] s_r = repeatability standard deviation.
[d] s_R= reproducibility standard deviation.
[e] RSD_r = repeatability relative standard deviation.
[f] RSD_r = reproducibility relative standard deviation.
[g] r = repeatability value (2.8 × s_r).
[h] R = reproducibility value (2.8 × s_R).

(**k**) *D-fructose standard stock solution.*—1.5 mg/mL in 0.2% benzoic acid. Before preparing solution, dry powdered crystalline fructose (purity > 97%) 16 h at 60°C under vacuum.

Items (**g**)–(**k**) are supplied in the Fructan Assay Kit available from Megazyme International Ireland Ltd., Bray Business Park, Bray, County Wicklow, Ireland, but preparations of enzymes and standards which meet these criteria may also be used.

D. Preparation of Test Samples, Standards, Reagent Blank, and Sucrose Control Powder

(**a**) *Test samples.*—For dry food samples, grind ca 50 g sample in grinding mill to pass 0.5 mm sieve. Transfer all material into wide-mouthed plastic jar and mix well by shaking and inversion. For solid moist samples such as chocolate, warm sample to room temperature (ca 22°C) and grate the material with a cheese grater. Take sample representing bulk material for analysis. For soft, very moist food samples such as low-fat spreads, warm material until it is soft; then stir product vigorously with a spatula and take a sample representing the bulk material. For liquid or semi-liquid samples such as juices or yogurt, adjust pH to ca 6.5 before heating. These samples can be directly diluted in the maleate buffer, *C*(**a**). All samples should be at room temperature (22°C) before they are weighed.

(**b**) *Fructose standard working solution*.—Add 0.2 mL fructose standard stock solution [1.5 mg/mL *C*(**k**)] to 0.9 mL acetate buffer, *C*(**b**), and mix thoroughly. Dispense 0.2 mL aliquots of this solution (containing 54.5 µg fructose) in quadruplicate to the bottoms of 4 glass test tubes, *B*(**m**). Add 0.1 mL acetate buffer, *C*(**b**), to each tube. Immediately before incubation in boiling water bath, add 5.0 mL PAHBAH working reagent, *C*(**c**).

(**c**) *Reagent blank*.—Transfer 0.3 mL acetate buffer, *C*(**b**), into test tubes and proceed with the standard assay procedure from *E*(**c**)(*3*).

(**d**) *Sucrose control powder*, *containing ca 10% sucrose.*—Analyze 1.0 g of this powder by the same procedure as used for samples containing 0–12% fructan. The sample contains no fructan and is used to check the effectiveness of the sucrase and borohydride treatments. The calculated fructan content should be no more than 0.3%.

E. Determination of Total Fructan Content of Samples

(**a**) *Extraction of fructan*.—(*1*) *Samples containing 0–12% fructan*.—Run D-fructose working standard solution (in quadruplicate), reagent blank (in duplicate), fructan control flour, and sucrose control flour with each set of samples. Use reagent blank to zero the spectrophotometer. (*i*) Accurately weigh 1.0 g sample into dry Pyrex beaker (200 mL capacity). Add 80 mL hot distilled water (~80°C); stir and heat beaker and contents on a hot-plate with a magnetic stirrer (at ~80°C) for ca 15 min (until sample is completely dispersed). It is important that pH of solution/slurry is above 5.5; otherwise fructan may be partially depolymerized. (*ii*) Cool solution to room temperature, and then quantitatively transfer to 100 mL volumetric flask; adjust volume to mark with distilled water, and mix. (*iii*) Filter aliquot of solution, *B*(**h**), and analyze immediately. Filtrate may be slightly turbid, depending on the nature of the sample extracted. This is not a problem. (If filtrate is stored for several hours at low temperature before analysis, the fructan may precipitate from solution. In such cases,

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reheat solution to ~80°C and let cool to room temperature before removing samples for analysis).

(*2*) *Samples containing 12–50% fructan.*—Run D-fructose working standard solution (in quadruplicate), reagent blank (in duplicate), fructan control flour, and sucrose control flour with each set of samples. Use reagent blank to zero the spectrophotometer. (*i*) Accurately weigh 90–100 mg ground test sample directly into dry Pyrex beaker (100 mL) capacity. Add 40 mL hot distilled water (~80°C); stir and heat beaker and contents on hot-plate with a magnetic stirrer (at ~80°C) for ca 15 min (until the sample is completely dispersed). It is important that pH of solution/slurry is above 5.5; otherwise fructan may be partially depolymerized. (*ii*) Cool solution to room temperature and then quantitatively transfer to 50 mL volumetric flask; adjust volume to mark with distilled water, and mix contents thoroughly. (*iii*) For samples containing 50–100% fructan, transfer solution to 100 mL volumetric flask and adjust to mark. (*iv*) Filter an aliquot of solution, *B*(**h**), and analyze immediately.

(**b**) *Removal of sucrose, starch, and reducing sugars.*—(*1*) Transfer 0.2 mL filtrate to be analyzed (containing ca 0.1–1.0 mg/mL fructan, or controls) to bottoms of 2 glass test tubes *B*(**m**).

(*2*) Add 0.2 mL diluted sucrase/amylase solution, *C*(**g**), to each tube and incubate at 40°C for 30 min.

(*3*) Add 0.2 mL alkaline borohydride solution, *C*(**e**), to each tube. Stir tube vigorously and incubate at 40°C for 30 min for complete reduction of reducing sugars to sugar alcohols.

(*4*) Add 0.5 mL acetic acid (100 mM), *C*(**f**), to each tube with vigorous stirring on Vortex mixer. If borohydride is fresh, a vigorous effervescence should be observed. If not, there is a problem with the borohydride, and the analysis will have to be repeated with fresh borohydride. (This treatment removes excess borohydride and adjusts pH to ca 4.5). This is termed Solution A.

(**c**) Hydrolysis and measurement of fructan.—(*1*) Transfer 0.2 mL aliquots Solution A (in duplicate) to the bottoms of glass test-tubes, *B*(**m**).

(*2*) Add 0.1 mL fructanase solution, *C*(**h**), to each test-tube, stir contents on Vortex mixer, and incubate tubes at 40°C for 20 min for complete hydrolysis of fructan to fructose and glucose.

(*3*) Treat contents of all tubes, including fructose standard working solution, *D*(**b**), reagent blank, *D*(**c**), and extracts of fructan control sample, *C*(**i**), and sucrose control sample, *C*(**j**), with PAHBAH working reagent (5.0 mL), and incubate in boiling water bath for exactly 6 min.

(*4*) Remove all tubes from boiling water bath and immediately place in cold water (18°–20°C) for ca 5 min.

(*5*) Measure absorbance of all solutions at 410 nm against reagent blank as soon as possible after cooling. The PAHBAH color complex will fade with time. At room temperature, little change (< 5%) is seen over 10–15 min. The same change will be seen in the standard solutions.

F. Calculations

Calculate total fructan content (%, on as-is basis) in test samples as follows:

$$\text{Total fructan, \%} = A \times F \times 5 \times V \times \frac{1.1}{0.2} \times \frac{100}{W} \times \frac{1}{1000} \times \frac{162}{180}$$

where: A = PAHBAH absorbance of reaction solutions (0.2 mL) read against 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 (100 or 50 mL); 1.1/0.2 = 0.2 mL was taken from 1.1 mL enzyme digest for analysis; W = weight (mg) of sample extracted; $100/W$ = factor to express fructan as percentage of flour weight; 1/1000 = factor to convert from μg to mg; 162/180 = factor to convert from free fructose, as determined, to anhydrofructose (and anhydroglucose), as occurs in fructan.

Indicative Controls

Indicative controls are used as a check on assay conditions. In this study, the sucrose/α-cellulose material was assayed under normal conditions. The final absorbance should be very low (< 0.03), which demonstrates the effectiveness of both the sucrase treatment step and the borohydride reduction step. If sucrose is not completely hydrolyzed by the sucrase treatment, it will then be hydrolyzed by the fructanase mixture and give erroneously high fructan values. Other indicative controls can be used, such as soluble starch and α-cellulose. The absorbance from α-cellulose should be negligible, i.e., < 0.001; for soluble starch, the absorbance should be very low, i.e., < 0.03. This result for starch demonstrates the effectiveness of the borohydride reduction step.

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Results and Discussion

Of the 15 laboratories provided with the method and materials, results from 2 participants were not included in final statistical calculations. One laboratory was excluded because of a basic flaw in chemicals used. From the results with the sucrose/α-cellulose control material, it was apparent that the borohydride used had degraded, i.e., the glucose and fructose released from sucrose by sucrase were not reduced to sugar alcohols, and thus were measured as fructan. Because most test materials contained some sucrose, most results reported were incorrect. The analyst was made aware of this problem but did not have time to repeat the study. The second laboratory was excluded when it was found to be an overall outlier laboratory with statistical outliers for 6 of the 9 pairs of test materials. This was after the Associate Referee had supplied materials to allow repeat analyses to be performed.

Helpful comments were made by several collaborators and most have been incorporated into the present procedure. For example: check pH of sample extract to ensure it was not sufficiently low to risk hydrolysis of fructans; clarifying the indi-

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Table 1. Determination of total fructan in food products and plant materials (g/100 g material) analyzed (part 1)

Sample	Chocolate		Milk powder		Vitamin tablet		Onion		Fructan control	
Code	A	M	B	F	C	E	D	O	G	R
Laboratory No.										
1	15.7	15.2	11.6	11.3	4.9	5.4	57.1	49.2	29.9	27.7
2	15.0	15.4	11.3	13.6	4.9	5.1	56.1	55.3	28.1	28.7
3	15.0	13.9	10.5	11.3	4.9	4.8	52.3	53.1	29.5	28.1
4	15.0	11.7	10.8	13.1	4.7	4.9	38.7[a]	38.3[a]	28.1	30.4
5	13.8	15.0	11.1	10.8	4.9,	4.9	53.3	53.1	29.6	30.0
6	13.6	13.8	11.2	10.9	4.7	4.8	51.6	53.1	29.6	30.1
7	13.3	13.5	10.5	9.4	4.5	3.3	48.5	49.6	25.4	25.9
8	13.1	14.5	10.5	10.1	4.4	4.4	42.4[b]	60.9[b]	29.8	36.5
9	13.0	12.9	10.8	10.7	5.2	4.5	51.2	45.3	26.9	28.4
10	12.4	12.2	10.0	9.8	4.4	4.5	49.3	48.3	28.3	28.6
11	12.3	12.1	9.0	9.6	4.1	4.0	48.3	47.6	27.3	28.2
12	12.3	14.1	10.2	10.4	4.2	4.9	55.0	51.4	27.2	29.1
13	11.1	11.5	9.1	9.0	4.1	4.1	46.4[a]	35.1[a]	27.0	21.7
No. labs[c]	13		13		13		10		13	
No. outliers[d]	0		0		0		3		0	
Mean	13.52		10.64		4.60		51.43		28.47	
s_r[e]	0.87		0.72		0.33		2.41		1.89	
s_R[f]	1.34		1.10		0.46		3.18		2.48	
RSD_r[g]	6.4		6.7		7.1		4.7		6.6	
RSD_R[h]	9.9		10.4		9.9		6.2		8.7	
r[i]	2.4		2.0		0.9		6.8		5.3	
R[j]	3.7		3.1		1.3		8.9		7.0	

Sample	Chocolate		Milk powder		Vitamin tablet		Onion		Fructan control	
Code	A	M	B	F	C	E	D	O	G	R
Duplicate assay RMS value	0.56	0.45	0.50	1.17	0.37	0.31	2.55	2.09	0.86	1.02

[a] Grubbs outlier.
[b] Cochran outlier.
[c] No. labs = number of laboratories included in calculations.
[d] No. outliers = number of outlier laboratories, not included in calculations.
[e] s_r = repeatability standard deviation.
[f] s_R= reproducibility standard deviation.
[g] RSD_r = repeatability relative standard deviation.
[h] RSD_R = reproducibility relative standard deviation.
[i] r = repeatability value ($2.8 \times s_r$).
[j] R = reproducibility value ($2.8 \times s_R$).

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Table 2. Determination of total fructan in food products and plant materials (g/100 g material) analyzed (part 2)

Sample	Pure fructan		Wheat stalks		Jerusalem artichoke		Low- fat spread	
Code	H	P	I	L	J	N	S	T
Laboratory No.								
1	99.6	96.4	5.0	5.1	55.8	53.6	8.6	10.1
2	97.3	100.0	5.4	5.1	53.0	57.6	9.1	9.3
3	99.4	97.7	4.5	4.5	50.4	51.8	8.2	8.3
4	94.2	91.2	4.4	4.4	47.1	52.9	6.9	9.1
5	102.9[a]	108.0[a]	5.1	5.1	52.7	57.6	9.0	8.8
6	99.6	103.5	4.9	4.9	51.7	53.7	8.6	8.8
7	88.9	95.3	4.7	4.7	48.8	51.2	7.9	8.4
8	105.8	NR[b]	4.0	4.1	49.2	50.2	8.9	8.2
9	94.8	91.4	4.7	4.6	47.7	53.5	7.9	7.9
10	100.2	104.1	4.9	4.7	49.8	49.2	8.2	7.7
11	93.0	88.9	5.1	5.5	47.6[a]	45.7[a]	6.5	6.5
12	89.3	92.6	4.6	4.6	49.4	48.9	7.9	7.2
13	67.4[a]	65.8[a]	4.1	4.1	35.0[a]	33.4[a]	7.1	7.8
No. labs[c]	10		13		11		13	
No. outliers[d]	2		0		2		0	
Mean	95.87		4.72		51.63		8.19	
S_r[e]	2.65		0.11		2.44		0.59	
S_r[f]	4.75		0.41		2.95		0.88	
RSD_r[g]	2.8		2.3		4.7		7.3	
RSD_R[h]	5.0		8.6		5.7		10.8	
r[i]	7.4		0.3		6.8		1.7	
R[j]	13.3		1.1		8.3		2.5	

Sample	Pure fructan		Wheat stalks		Jerusalem artichoke		Low-fat spread	
Code	H	P	I	L	J	N	S	T
Duplicate assay RMS value	2.29	1.35	0.28	0.13	1.21	1.41	0.34	0.41

[a] Grubbs outlier.
[b] NR = not reported.
[c] No. labs = number of laboratories included in calculations.
[d] No. outliers = number of outlier laboratories, not included in calculations.
[e] s_r = repeatability standard deviation.
[f] s_R= reproducibility standard deviation.
[g] RSD_r = repeatability relative standard deviation.
[h] RSD_R = reproducibility relative standard deviation.
[i] r = repeatability value ($2.8 \times s_r$).
[j] R = reproducibility value ($2.8 \times s_R$).

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cations that borohydride reagent may have degraded; making extract solutions to volume, rather than using pre-weighed beakers. Additional suggestions were made concerning inclusion of blank, standard, and controls through all steps of the procedure.

Collaborators' data were evaluated statistically according to AOAC protocols using AOAC-supplied software. Cochran (repeatability) and Grubbs (reproducibility) outlier tests revealed 7 outlier results as follows: laboratory 13 had 3 Grubbs (low) outlier results, one each for onion, pure fructan, and Jerusalem artichoke, respectively. Laboratories 4, 5, and 11 had one Grubbs outlier each for onion, pure fructan, and Jerusalem artichoke, respectively. Laboratory 8 had one Cochran outlier for onion. Data with calculated means and precision values are shown in Tables 1 and 2. The average fructans contents ranged from 4.60% for vitamin tablet to 95.87% for pure fructan material. All materials had repeatability relative standard deviations (RSD_r) < 8%, with 4 materials having RSD_r of < 5%. The reproducibility relative standard deviation (RSD_R) varied from 5% for pure fructan to 10.8% for low-fat spread, with 7 of the 9 test samples having an RSD_R of <10%. The final statistical data were calculated from 218 determinations on 9 foods, of which 14 determinations were outliers.

Participating laboratories were requested to perform single determinations on each (blind duplicate) material, but duplicate assays on aliquots of each test material extract were also requested. Only laboratory 11 did not supply results for duplicate aliquots, and laboratory 5 supplied triplicate results. The variability of these duplicate assays was calculated by the root mean square (RMS) value of the one-way analysis of variance. It is interesting to compare these RMS values with repeatability standard deviations (s_r), because the variance from duplicate aliquot assays contributes to the size of s_r values. RMS calculations were made separately for each test material (Tables 1 and 2), and for each laboratory (Table 3). From Table 1 it is evident that duplicate assay RMS values for each material were similar in magnitude, but less than the s_r values between blind duplicates for the materials milk powder, vitamin tablets, onion, and wheat stalks. RMS values were approximately half the s_r values for chocolate, fructan control, pure fructan, Jerusalem artichoke, and low-fat spread. The variability indicated by these RMS values can be attributed mainly to errors in pipetting or possibly to inhomogeneity of test sample extracts. Both of these sources of error are common problems in technique. Analysts should be aware that accuracy and precision of pipettors cannot be assumed and that these can easily be verified before use by checking the mass of repeated aliquots of pure water on an analytical balance. Pipettor accuracy and precision should both lie within manufacturers' specifications. Homogenization by mixing or stirring is fundamental to reliable analysis for solutions being subsampled and for weighing the initial test portion.

RMS values for each laboratory were calculated separately for lower and higher fructan-containing materials, as well as all materials. These values give an indication of individual laboratory performance, mainly with regard to pipetting proficiency and extract homogeneity. Calculated RMS values were ≤0.5 for laboratories 4, 6, 7, 9, and 13. RMS values were between 0.5 and 1.0 for laboratories 3, 8, and 10; between 1.0 and 2.0 for laboratories 5 and 12, and >2.0 for laboratory 2. These RMS values indicate how much improvement may be possible for repeatability and reproducibility of this method (and other similar methods).

Comparison of the relative precision (RSD_R, %) of the current enzymatic procedure for measuring fructan with that of

Table 3. Laboratory precision between duplicate aliquots for assay

Laboratory No.	Precision between duplicate aliquots (RMS)[a]		
	Lower fructan materials	Higher fructan materials	For all materials
1	0.18	1.93	1.23
2	1.48	3.89	2.71
3	0.15	1.04	0.67
4	0.35	0.74	0.54
5	0.12	2.94	1.86
6	0.24	0.55	0.41
7	0.10	0.79	0.51
8	0.35	1.27	0.79
9	0.30	0.76	0.53
10	0.19	1.24	0.80
11	—	—	—
12	0.01	2.68	1.70
13	0.15	0.68	0.44

[a] RMS = root mean square.

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the instrumental/enzymatic method (5; AOAC Method **997.08**) shows similar RSD_R values for samples containing 1–40% fructan. The current method is easy to use and requires no specialized equipment. A specific sucrase enzyme is used to remove sucrose, whereas in the instrumental method, this is allowed for after chromatographic separation. The specific sucrase could be used in the instrumental method to reduce the number of steps required. Also, in the current procedure, a mixture of highly purified *exo*- and *endo*-inulinanases are used. These have been chromatographically purified to remove α-galactosidase, and significantly reduce pectinase and β-glucanase (cellulase). Consequently, this enzyme mixture (fructanase) can be used to specifically hydrolyze fructans in mixtures containing pectin and β-glucan. The presence of significant levels of pectinase and/or β-glucanase in fructanase preparations (as in commercial Fructozyme® preparation; Novozym SP 230®, Novo Nordisk, SA, France) precludes the use of the preparation in an integrated method for measuring fructan and the AOAC total dietary fiber methods. Pectinase can be effectively removed by heat treatment (7), but removal of β-glucanase requires chromatographic purification (6). With the purified fructanase preparation used in the current procedure, it is possible to integrate fructan (inulin) determination in the AOAC methods for measuring total dietary fiber.

Recommendation

On the basis of the results of this study, it is recommended that the spectrophotometric method for total fructans determination in food and food products be adopted First Action.

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