FOOD COMPOSITION AND ADDITIVES

Total Dietary Fiber (CODEX Definition) in Foods and Food Ingredients by a Rapid Enzymatic-Gravimetric Method and Liquid Chromatography: Collaborative Study, First Action 2017.16

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A method for measurement of total dietary fiber (TDF) has been validated. This method is applicable to plant materials, foods, and food ingredients as consumed, consistent with the 2009 CODEX definition (ALINORM 09/32/REP), and measures insoluble dietary fiber (IDF) and soluble dietary fiber (SDF), comprising SDF that precipitates in the presence of 78% ethanol (SDFP) and SDF that remains soluble in the presence of 78% ethanol (SDFS). The method is an update of AOAC Method 2009.01 and addresses each of the issues identified by analysts in using that method over the past 8 years. A total of 13 laboratories participated in the study, with all laboratories returning valid assay data for most of the 16 test portions (8 blind duplicates) consisting of samples with a range of content of traditional dietary fibers, resistant starch, and nondigestible oligosaccharides. The dietary fiber content of the eight test pairs ranged from 6.90 to 60.37 g/100 g. TDF was calculated as the sum of IDF plus SDFP measured gravimetrically and SDFS measured by HPLC. The repeatability SD ranged from 0.27 to 0.76 g/100 g, and the reproducibility SD ranged from 0.54 to 3.99 g/100 g. The RSD_r ranged from 1.22 to 6.52%, and the RSD_R ranged from 2.14 to 10.62%.

The definition for dietary fiber adopted by the Codex Alimentarius Commission (CAC) in June 2009 (1) includes carbohydrate polymers that are not hydrolyzed by the endogenous enzymes in the small intestine of humans and thus includes resistant starch (RS). This definition also includes oligosaccharides of degrees of polymerization (DP) 3–9, but the decision on whether to include these oligosaccharides in the dietary fiber value was left to the discretion of national authorities.

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This method was approved by the AOAC Expert Review Panel for Total Dietary Fiber as First Action.
The Expert Review Panel invites method users to provide feedback on the First Action methods. Feedback from method users will help verify that the methods are fit-for-purpose and is critical for gaining global recognition and acceptance of the methods. Comments can be sent directly to the corresponding author or methodfeedback@aoac.org.
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A method designed to support the CAC definition was published in 2007 (2), and this method was successfully evaluated in interlaboratory studies (3, 4) and approved by AOAC INTERNATIONAL (**2009.01**; **2011.25**) (3–5). AOAC Method **2009.01** allows the measurement of TDF by summing the quantity of higher molecular weight dietary fiber, which included insoluble dietary fiber (IDF) and soluble dietary fiber (SDF) that precipitates in the presence of 78% aqueous ethanol (SDFP), with SDF that remains soluble in 78% aqueous ethanol (SDFS). However, application of this method to a range of food products and ingredients over the past 8 years has identified several challenges/concerns:, (*1*) An incubation time with pancreatic α-amylase (PAA) plus amyloglucosidase (AMG) of 16 h (the digestion step paralleling the incubation conditions employed in AOAC **2002.02** for RS; 4) has no physiological basis. A more likely residence time for food in the small intestine is 4 ± 1 h (6, 7, and references therein). (*2*) Most commercially available fructo-oligosaccharides (FOS) contain the trisaccharide fructosyl-β-(2-1)-fructosyl-β-(2-1)-fructose (inulinotriose), which is not measured as dietary fiber using a Waters Sugar-Pak® HPLC column because it elutes with the disaccharide fraction. (*3*) Under the incubation conditions used, resistant maltodextrins are produced during the hydrolysis of nonresistant starch, and these are incorrectly measured as dietary fiber (6, 8, 9). (*4*) The extended incubation time of samples with PAA/AMG results in excessive hydrolysis, and thus underestimation, of phosphate cross-linked starch (RS_4, e.g., Fibersym; 10). (*5*) The use of the preservative sodium azide is undesirable on the basis of health concerns to analysts.

The procedure described here, a "rapid" integrated TDF (RINTDF) method, employs the same basic biochemistry and enzymes (PAA, AMG, and protease) as used in AOAC **2009.01**, but it resolves each of the challenges detailed above, and in particular, with an incubation time of 4 h, it more closely simulates physiological conditions in the human small intestine. More accurate measurement of FOS is achieved by performing HPLC of SDFS with TSKgel®$G2500PW_{XL}$ gel permeation columns (11) with in-line deionization (12).

Collaborative Study

Precollaborative Ruggedness Testing

A precollaborative ruggedness study was conducted with all the participating laboratories to ensure adequate method performance. Volunteer laboratories were sent four samples along

with copies of the method, required enzymes, control solutions, and the required ion exchange resins. Each laboratory was asked to run a single analysis of each sample, to ask questions regarding the procedures, and to provide feedback to the method author. The results of the analysis on the ruggedness testing samples are shown in Table 1. In the gravimetric determinations of IDF + SDFP, no specific problems were identified by the collaborators.

Table 1. Study data for the precollaborative evaluation of the RINTDF method for total dietary fiber

	Total dietary fiber			
Lab No.	A[a]	B[b]	C[c]	D[d]
1	12.46	62.32	20.46	22.24
2	10.93	66.0	19.34	21.5
3	9.95	51.32	18.56	21.88
4	13.1	62.9	19.7	23.2
5	11.40	60.39	19.64	23.23
6	11.48	58.95	18.00	21.25
7	12.53	62.67	21.00	21.22
8	13.04	57.74	19.52	22.17
9	13.04	60.46	19.79	22.78
10	12.34	61.17	19.57	22.74
11	11.57	59.83	19.63	23.57
12	12.28	60.23	20.97	16.85
13	9.86	60.38	16.58	20.07
Mean of lab avg., g/100 g	11.85	60.33	19.44	21.75
s_R, g/100 g	1.10	3.40	1.20	1.77
RSD_R, %	9.28	5.64	6.15	8.13

[a] A = Whole meal bread.

[b] B = High-amylose maize starch (Hylon VII).

[c] C = Carrots lyophilized.

[d] D = Heinz baked beans washed and lyophilized.

Some problems and misunderstandings occurred in the measurement of the SDFS fraction by HPLC, i.e., the importance of using the stated HPLC column, correct deionization of samples, and proper maintenance of the HPLC columns. The importance of maintaining the sample in suspension during the incubation with PAA/AMG was again highlighted. Also, some error was introduced in the preparation of the D-glucose/glycerol standard solutions. It was thus decided to provide this solution in a ready-to-use form for the full collaborative study.

The results of the precollaborative study were typical for dietary fiber methods. Repeatability, reproducibility, and the Horwitz ratio (HorRat) values were within the range of performance characteristics typically found for dietary fiber methods, wherein a significant number of manual steps are necessary to perform the assay. Samples were analyzed for IDF + SDFP gravimetrically and SDFS by HPLC. The reproducibility SD (s_R) ranged from 1.10 to 3.40 g/100 g, and the RSD_R ranged from 5.64 to 9.28%, values consistent with those reported for analyses of similar samples with other dietary fiber assay formats (Table 2).

The method author thus determined that the method was ready for a full collaborative study.

Collaborative Study Protocol

Eight food samples were selected for the collaborative study, and because the main focus of the study was to evaluate complex food samples containing resistant starch and nondigestible oligosaccharides, samples high in these components were chosen. These samples included legumes, RS_4, whole-grain products, and food products enriched with resistant starch and nondigestible oligosaccharides. The inclusion of resistant starch and nondigestible oligosaccharides in the CAC definition of dietary fibers dictates that updated testing procedures must include an accurate measure of these components and offer analytical protocols that are as user friendly as possible while accurately and reliably measuring the components of interest.

Table 2. Comparable AOAC INTERNATIONAL method data[a]

Method	Title	s_r, g/100g	RSD_r, %	s_R, g/100g	RSD_R, %	HorRat
AOAC **985.29**	Total Dietary Fiber in Foods	0.15–0.99	0.56–66.25	0.27–1.36	1.58–66.25	0.76–17.46
AOAC **991.42**	Insoluble Dietary Fiber in Foods and Food Products	0.41–2.82	0.86–10.38	0.62–9.49	3.68–19.44	1.73–8.68
AOAC **991.43**	Soluble, Insoluble, and Total Dietary Fiber in Foods and Food Products	0.36–1.06	1.50–6.62	0.41–1.43	1.58–12.17	0.74–4.66
AOAC **992.16**	Total Dietary Fiber	0.18–1.01	1.48–14.73	0.22–2.06	4.13–17.94	1.84–4.62
AOAC **993.19**	Soluble Dietary Fiber in Foods and Food Products	0.49–1.15	1.74–5.93	0.79–2.05	2.41–7.01	1.13–2.83
AACC **994.13**	Total Dietary Fiber (Determined as Neutral Sugar Residues, Uronic Acid Residues and Klason Lignin)	0.32–2.88	1.80–6.96	0.52–4.90	4.80–11.30	2.32–4.20
AOAC **2001.03**	Dietary Fiber Containing Supplemented Resistant Maltodextrin (RMD)	0.02–1.63	1.33–6.10	0.04–2.37	1.79–9.39	0.77–3.32
AOAC **2002.02**	Resistant Starch in Starch and Plant Materials	0.08–2.66	1.97–4.12	0.21–3.87	4.48–10.90	1.44–3.74
AOAC **2009.01**	Total Dietary Fiber in Foods	0.41–1.43	1.65–12.34	1.18–5.44	4.70–17.97	1.91–6.49
AOAC **2011.25**	Insoluble, Soluble and Total Dietary Fiber (Codex Definition) by an Enzymatic-Gravimetric Method and Liquid Chromatography	0.47–1.41	2.43–8.60	0.95–3.14	6.85–14.48	2.85–5.51
RINTDF Method	Total Dietary Fiber in Foods (Codex Definition) by a Rapid Enzymatic-Gravimetric Method and Liquid Chromatography[b]	0.27–0.76	1.22–6.52	0.54–3.99	2.14–10.62	1.08–4.46

[a] Samples were not dried and/or were desugared only.

[b] Current method.

Moist samples were freeze-dried. All samples were ground to the method-specific size, homogenized, and mixed thoroughly before being subdivided into glass vials that were then sealed and capped. Samples, copies of the method, electronic report sheets, Excel-based calculators, sample storage instructions, and an adequate supply of enzymes, reference standards, and resins were distributed to collaborating laboratories using express overnight shipment.

The same 13 laboratories that were involved in the precollaborative ruggedness testing completed the study and reported a full set of results. Of these, two laboratories did not have the required HPLC columns and were unable to perform these analyses in-house. Consequently, the SDFS fraction of the samples produced by these two laboratories were concentrated according to the provided method and sent to the laboratory of the method author by express overnight shipment, where they were deionized and analyzed for SDFS. Results were returned to the collaborator for calculation of TDF as IDF + SDFP (gravimetrically) and SDFS (by HPLC). It was subsequently realized that the involvement of the method author's lab in any aspect of the analysis of samples from an external collaborator is unorthodox, so the data supplied by these two laboratories (laboratories 2 and 12; Table 3) have been excluded from the statistical analysis (Table 4). However, as a sign of appreciation to those collaborators, a separate table of statistics that includes the data supplied by these two laboratories is also shown (Table 5). The repeatability, reproducibility, s_r, and s_R values obtained in analyzing data from the 13 laboratories were very similar to those obtained in the analysis of the data from the 11 laboratories that form the basis of this validation.

Statistical Treatment

Collaborating laboratory data were evaluated statistically according to AOAC INTERNATIONAL protocols using AOAC-supplied software. Of the 88 valid pairs of assay results reported from 11 laboratories (laboratories 2 and 12 excluded) for TDF, laboratories 1, 4, 5, 6, 7, 8, 9, 10, and 11 had no statistical outliers and laboratories 3 and 13 had one statistical outlier, for a total of two statistical outlier pairs overall. The raw data and statistically paired data from the blind duplicate results for TDF reported by the collaborating laboratories are shown in Tables 3 and 4, respectively. Outliers and the reason for outlier removal are indicated and footnoted in Table 3.

AOAC Official Method 2017.16
Total Dietary Fiber in Foods and Food Ingredients Rapid Integrated Enzymatic-Gravimetric–High Pressure Liquid Chromatography Method

[Applicable to plant material, foods, and food ingredients.]

A. Principle

A method is described for the measurement of TDF as defined by the CAC. This method quantitates TDF by gravimetric and HPLC procedures (Figure **2017.16A**). RS is captured in the IDF fraction. This method combines the key attributes of AOAC *Official Methods of Analysis* **985.29** (13), **2001.03** (11), and

Table 3. Interlaboratory study results for total dietary fiber in foods (RINTDF Method)

Sample/ Lab	Total dietary fiber, g/100 g															
	A & D[a]		B & F[b]		C & J[c]		E & H[d]		G & N[e]		I & M[f]		K & O[g]		L & P[h]	
1	58.70	59.44	25.26	24.42	30.82	29.56	6.07	6.31	16.91	17.33	19.59	19.91	21.03	21.23	10.36	10.60
2	68.04	69.30	23.82	25.67	30.64	31.19	7.19	7.97	17.26	17.24	21.84	21.48	22.23	21.29	10.00	10.71
3	55.02	54.89	24.11	24.48	28.52	28.73	8.16[i]	6.40[i]	15.12	14.21	17.49	18.38	21.60	21.65	11.84	10.45
4	62.17	61.36	23.87	22.92	28.70	28.40	6.73	6.74	15.42	15.32	19.45	19.74	20.00	21.15	11.29	11.38
5	62.07	62.25	23.46	24.46	29.26	29.21	7.21	6.79	15.94	16.30	18.78	18.69	20.74	20.67	10.49	11.33
6	62.37	62.94	23.02	23.23	29.42	29.59	7.15	6.44	16.40	16.52	20.12	20.40	21.22	21.46	10.37	10.52
7	67.56	69.00	23.78	23.88	28.74	28.90	6.39	6.45	15.78	15.86	17.83	17.12	21.17	20.14	11.44	10.81
8	56.91	55.42	22.78	24.39	28.88	29.34	6.31	6.32	16.26	15.35	20.28	20.05	20.52	19.83	8.98	9.39
9	62.83	60.75	24.49	24.66	30.16	30.12	8.10	8.34	17.37	16.71	20.13	20.66	21.80	21.54	11.60	12.32
10	56.43	56.00	23.61	23.79	29.53	29.69	7.36	7.86	16.46	16.79	18.45	18.41	21.02	21.32	10.20	12.79
11	61.16	60.12	22.25	23.69	29.17	28.39	6.40	5.79	16.75	16.47	21.13	21.08	21.14	21.13	11.25	10.61
12	54.98	55.28	21.81	21.37	31.90[i]	28.07[i]	4.88	5.03	15.16	14.86	15.01	15.33	18.24[j]	18.66[j]	10.11	10.91
13	65.83[i]	61.37[i]	23.19	23.82	28.70	29.11	7.54	7.65	16.56	15.45	20.03	19.79	21.44	20.74	9.92	10.03

[a] A & D = Phosphate cross-linked starch (Fibersym).

[b] B & F = Kidney beans (canned, washed, and lyophilized).

[c] C & J = Bran cereal.

[d] E & H = Defatted cookies containing FOS.

[e] G & N = Oat bran.

[f] I & M = Defatted cookies containing polydextrose and RS_2.

[g] K & O = Dark rye crispbread.

[h] L & P = Whole meal bread.

[i] Removed on basis of Cochran's Test.

[j] Removed on basis of the lowest average in the Single Grubb's Test.

Table 4. Interlaboratory study results for total dietary fiber in foods (RINTDF Method) in which data from laboratories 2 and 12 were excluded; statistical evaluation according to AOAC INTERNATIONAL statistics format

Sample/parameter	A & D[a]	B & F[b]	C & J[c]	E & H[d]	G & N[e]	I & M[f]	K & O[g]	L & P[h]
No. labs/analysts	10	11	11	10	11	11	11	11
Mean, g/100 g	60.37	23.80	29.22	6.90	16.15	19.43	21.02	10.82
s_r, g/100 g	0.76	0.60	0.36	0.27	0.42	0.30	0.41	0.71
s_R, g/100 g	3.99	0.72	0.63	0.73	0.79	1.15	0.54	0.91
RSD_r, %	1.25	2.52	1.22	3.88	2.59	1.54	1.93	6.52
RSD_R, %	6.62	3.03	2.14	10.62	4.88	5.94	2.57	8.46

[a] A & D = Phosphate cross-linked starch (Fibersym).

[b] B & F = Kidney beans (canned, washed, and lyophilized).

[c] C & J = Bran cereal.

[d] E & H = Defatted cookies containing FOS.

[e] G & N = Oat bran.

[f] I & M = Defatted cookies containing polydextrose and RS_2.

[g] K & O = Dark rye crispbread.

[h] L & P = Whole meal bread.

Table 5. Interlaboratory study results for total dietary fiber in foods (RINTDF Method) in which data from all 13 participating laboratories were included; statistical evaluation according to AOAC INTERNATIONAL statistics format

Sample/parameter	A & D[a]	B & F[b]	C & J[c]	E & H[d]	G & N[e]	I & M[f]	K & O[g]	L & P[h]
No. labs/analysts	12	13	12	12	13	13	12	13
Mean, g/100 g	60.62	23.70	29.37	6.79	16.15	19.28	21.09	10.76
s_r, g/100 g	0.74	0.67	0.36	0.29	0.39	0.29	0.43	0.68
s_R, g/100 g	4.67	0.99	0.78	0.91	0.85	1.74	0.57	0.86
RSD_r, %	1.22	2.81	1.22	4.32	2.41	1.51	2.05	6.34
RSD_R, %	7.70	4.17	2.64	13.38	5.29	9.01	2.72	8.02

[a] A & D = Phosphate cross-linked starch (Fibersym).

[b] B & F = Kidney beans (canned, washed, and lyophilized).

[c] C & J = Bran cereal.

[d] E & H = Defatted cookies containing FOS.

[e] G & N = Oat bran.

[f] I & M = Defatted cookies containing polydextrose and RS_2.

[g] K & O = Dark rye crispbread.

[h] L & P = Whole meal bread.

2002.02 (4) and is an update of AOAC Method **2009.01** (2–4). Duplicate test portions are incubated with PAA and AMG for 4 h at 37°C in sealed 250 mL bottles in a shaking water bath while mixing in orbital motion, or stirring with a magnetic stirrer, during which time nonresistant starch is solubilized and hydrolyzed to glucose and maltose by the combined action of the two enzymes. The reaction is terminated by pH adjustment followed by temporary heating. Protein in the sample is digested with protease. For the measurement of TDF, ethanol (EtOH) or industrial methylated spirits (IMS) are added, and the IDF and SDFP are captured on a sintered glass crucible, washed with EtOH and acetone, dried, and weighed. One of the duplicate residues is analyzed for protein, the other for ash. SDFS in the filtrate is concentrated, deionized with resins, and quantitated by HPLC. This method differs from AOAC **2009.01** in that incubation time with PAA and AMG is reduced from 16 to 4 h (with higher concentrations of enzymes used) to better simulate human intestinal residence time, improved deionization and HPLC separation of SDFS is incorporated, glycerol is used as the internal standard, and sodium azide is deleted from the incubation buffer.

B. Apparatus

(**a**) *Grinding mill*.—Centrifugal, with 12 tooth rotor and 0.5 mm sieve, or similar device. Alternatively, cyclone mill can be used for small test laboratory samples provided they have sufficient air flow or other cooling to avoid overheating samples.

(**b**) *Digestion bottles*.—250 mL Fisherbrand soda glass, wide-mouth bottles with polyvinyl lined cap (Cat. No. 11798859; Fisher Scientific, Göteborg, Sweden; Figures **2017.16B** and **2017.16C**).

(**c**) *Fritted crucible*.—Büchner, fritted disk, Pyrex 50 mL, pore size coarse, American Society for Testing and Materials 40–60 μm (Product No. 32940-50C; Corning Life Sciences,

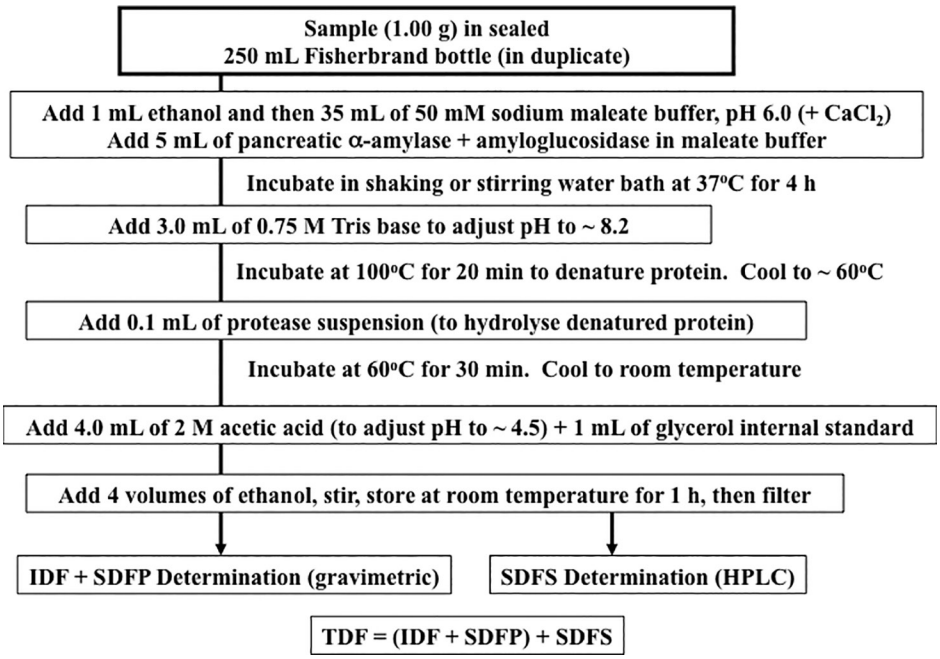

Figure 2017.16A. Rapid integrated total dietary fiber assay procedure showing the key steps in the procedure.

Tawksbury, MA). Prepare four for each sample as follows: ash overnight at 525°C in muffle furnace; cool furnace to 130°C before removing crucibles to minimize breakage. Remove any residual Celite and ash material by using a vacuum. Soak in 2% Micro cleaning solution, **C(o)**, at room temperature for 1 h. Rinse crucibles with water and deionized water. For final rinse, use 15 mL acetone and air dry. Add approximately 1.0 g Celite to dried crucibles and dry at 130°C to constant weight. Cool crucible in desiccators for approximately 1 hr and record weight of crucible containing Celite.

(**d**) *Filtering flask*.—Heavy-walled, 1 L with side arm.

(**e**) *Rubber ring adaptors*.—For use to join crucibles with filtering flasks.

(**f**) *Vacuum source*.—Vacuum pump or aspirator with regulator capable of regulating vacuum.

(**g**) *Water bath(s)*.—Rotary motion, shaking, large-capacity (20–24 L) with covers; capable of maintaining temperature of 37 ± 1 and 60 ± 1°C (Grant Instruments, Shepreth, Royston, United Kingdom). Ensure that shaking action/sample agitation in water bath is sufficient to maintain sample solids in suspension and that no residue buildup or rings of sample material form in the digestion bottle during the enzymatic digestions (i.e., at 150 rev/min; Figure **2017.16B**). If the water bath is used in linear motion (not preferred motion), then the bottles must be placed at an angle of 45° to the direction of movement to ensure continual suspension of the sample during the 4 h incubation period with PAA/AMG. Alternatively, mixing can be achieved with a 2mag Mixdrive 15® submersible magnetic stirrer with a 30 × 7 mm stirrer bar, set at 170 rpm (2mag AG, Munich, Germany; Figure **2017.16C**).

(**h**) *Balance*.—0.1 mg readability, accuracy, and precision.

(**i**) *Ovens*.—Two, mechanical convection, set at 103 ± 2 and 130 ± 3°C.

(**j**) *Timer*.

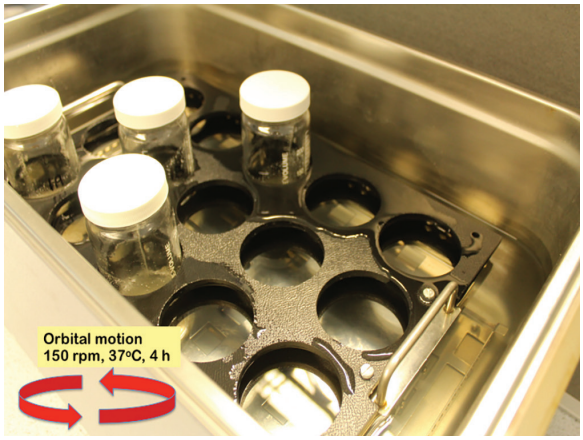

Figure 2017.16B. Incubation of samples in Fisherbrand incubation bottles in a shaking water bath showing custom-made polypropylene bottle holder.

Figure 2017.16C. 2mag Mixdrive 15 submersible magnetic stirrer in custom-built bath with Fisherbrand incubation bottles.

(**k**) *Desiccator*.—Airtight, with SiO_2 or equivalent desiccant. Desiccant dried biweekly overnight in 130°C oven, or more frequently as needed.

(**l**) *pH meter.*

(**m**) *Pipettors and tips*.—50–200 µL and 5 mL capacity.

(**n**) *Dispensers.—(1)15 ± 0.5 mL for 78% EtOH (or IMS), 95% EtOH (or IMS), and acetone.*

(*2*) *35 ± 0.2 mL buffer.*

(**o**) *Cylinder*.—Graduated, 100 and 500 mL.

(**p**) *Magnetic stirrers and stirring bars.*

(**q**) *Rubber spatulas.*

(**r**) *Muffle furnace*.—525 ± 5°C.

(**s**) *Polypropylene tube*.—13 mL, 101 × 16.5 mm, flat base with screw cap.

(**t**) *HPLC*.—With oven to maintain a column temperature of 80°C and a 50 µL injection loop. Column operating conditions: temperature, 80°C; mobile phase, distilled water, flow rate, 0.5 mL/min.

(**u**) *HPLC columns*.—TSKgel G2500PW_{XL} columns, 30 cm × 7.8 mm, connected in series. Operate at 80°C. Mobile phase: distilled water at 0.5 mL/min. System must be capable of separating maltose from maltotriose (Figure **2017.16D**). Run time of 60 min to ensure that all materials from the injection are cleared from the column prior to the next injection.

(**v**) *Cation and anion exchange guard column (containing deionizing/desalting cartridges)*.—Cation and anion exchange guard cartridges, H^+ and CO_2^{3-} forms, respectively (Cat. No. 125-0118; Bio-Rad Laboratories, Hercules, CA; includes one cation and one anion cartridge), with guard column holder (Cat. No. 125-039; Bio-Rad Laboratories) to hold the two guard cartridges in series, cation cartridge preceding anion cartridge.

(**w**) *Guard column (or precolumn)*.—TSKgel PW_{XL} guard column (TOSOH Corp., Tokyo, Japan).

(**x**) *Detector*.—Refractive index (RI); maintained at 50°C.

(**y**) *Data integrator or computer*.—For peak area measurement.

(**z**) *Filters for disposable syringe*.—0.45 µm membrane, 13 or 25 mm.

(**aa**) *Filters for water*.—Polyvinylidene fluoride, pore size 0.45 µm, 47 mm.

(**bb**) *Filter apparatus*.—To hold 47 mm, 0.45 µm filter, **B(aa)**, to filter larger volumes of water.

(**cc**) *Syringes*.—10 mL, disposable plastic.

(**dd**) *Syringes*.—Hamilton 100 µL, 710SNR syringe (Sigma-Aldrich Ireland Ltd, Arklow, Ireland).

(**ee**) *Rotary evaporator*.—Heidolph Laborota 4000 or equivalent (Heidolph, Elk Grove Village, IL).

(**ff**) *Thermometer*.—Capable of measuring to 100°C.

C. Reagents

(**a**) *EtOH 95%, v/v (or IMS)*.—IMS made up of EtOH 84.8333% (w/w), 85.952% (v/v); water 5.6571% (w/w), 4.524% (v/v); 2-propanol 4.9118% (w/w), 5.0000% (v/v); methanol 4.5979% (w/w), and 4.524% (v/v). It can be prepared by mixing 5 volumes of 2 propanol with 95 volumes of denatured ethanol formula SDA-3A (100 volumes of 95% EtOH combined with 5 volumes of methanol).

(**b**) *EtOH (or IMS), 78%*.—Place 179 mL water into 1 L volumetric flask. Dilute to volume with 95% EtOH or IMS. Mix.

(**c**) *Acetone*.—Reagent grade.

(**d**) *Stock PAA plus AMG powder*.—PAA (40 KU/g) plus AMG (17 KU/g) as a freeze-dried powder mixture. (*Note:* One Unit AMG activity is the amount of enzyme required to release one µmol D-glucose from soluble starch per minute at 40°C and pH 4.5; one Unit PAA activity is the amount of enzyme required to release one µmol *p*-nitrophenyl from Ceralpha reagent per min at 40°C and pH 6.9; AOAC **2002.01**). PAA/AMG preparations should be essentially devoid of β-glucanase, β−xylanase and detectable levels of free D-glucose. Stable for >4 years at –20°C.

(**e**) *PAA (4 KU/5 mL)/AMG (1.7 KU/5 mL)*.—Immediately before use, dissolve 1 g PAA/AMG powder in 50 mL sodium maleate buffer (50 mM, pH 6.0 plus 2 mM $CaCl_2$) and stir for approximately 5 min. Store on ice during use. Use on the day of preparation. *Alternatively*.—Some individuals are allergic

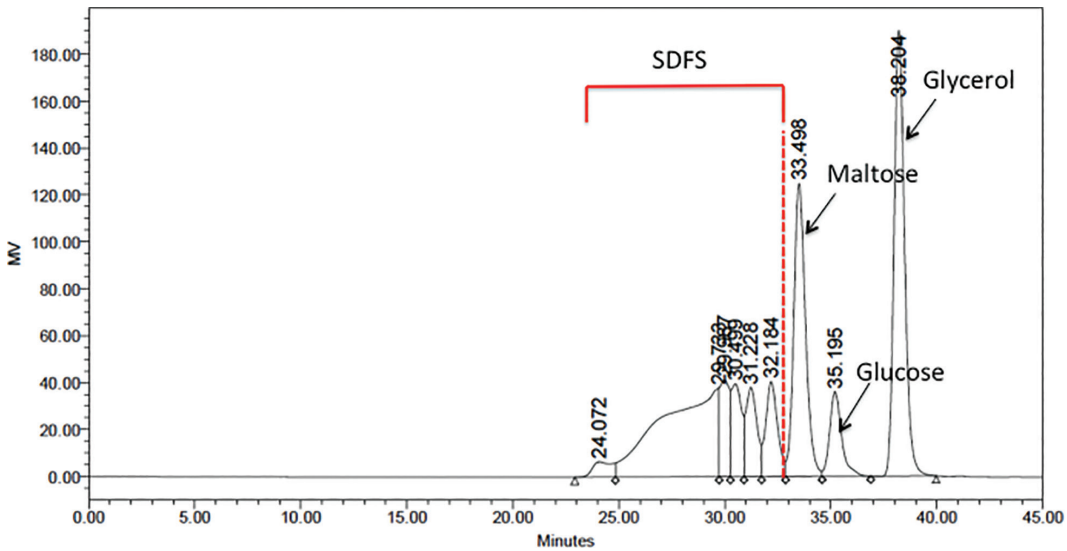

Figure 2017.16D. Chromatograms of a mixture of maltodextrins, glucose, and glycerol on two TSKgel G2500PWXL columns in series. Solvent: distilled water, flow rate: 0.5 mL/min, and temperature: 80°C. The dashed lines show demarcation between DP2 (maltose) and DP 3 (higher maltodextrins). The fraction shown as SDFS denotes the fraction that would be collected as SDFS; however, in this case, these are maltodextrins that would be hydrolyzed by the PAA/AMG mixture.

to powdered PAA and/or AMG. In this instance, engage an analyst who is not allergic to prepare the powdered enzymes as an ammonium sulphate suspension as follows: Gradually add 5 g PAA/AMG powder mix (PAA 40 KU/g plus AMG 17 KU/g; reagent 4) to 70 mL cold, distilled water in a 200 mL beaker on a magnetic stirrer in a laboratory hood and stir until the enzymes are completely dissolved (approximately 5 min). Add 35 g granular ammonium sulphate and dissolve by stirring. Adjust the volume to 100 mL with ammonium sulphate solution (50 g/100 mL) and store at 4°C. (This preparation contains PAA at 2 KU/mL and AMG at 0.85 KU/mL). Stable at 4°C for 3 months.

(**f**) *Protease suspension (50 mg/mL, approximately 6 Tyrosine U/mg)*.—Stabilized suspension in 3.2 M ammonium sulphate. Swirl gently before use. Dispense using a positive displacement dispenser. Protease must be devoid of α-amylase and essentially devoid of β-glucanase and β-xylanase. Use as supplied. Stable for >4 years at 4°C.

(**g**) *Glycerol internal standard*.—100 mg/mL containing sodium azide (0.02%, w/v). Stable for >4 years at 4°C. Diethyleneglycol (100 mg/mL) in sodium azide (0.02%) is an alternative internal standard. This is less stable than the glycerol standard, so must be prepared on a weekly basis.

(**h**) *LC retention time standard (maltodextrins)*.—Dissolve 1.25 g retention time standard consisting of corn syrup solids (DP ≥3) and maltose in 30 mL of 0.02% sodium azide solution and transfer to a 50 mL volumetric flask. Pipette 5 mL glycerol internal standard (100 mg/mL). Bring to 50 mL with 0.02% sodium azide solution **C(n)**. Transfer solutions to 50 mL Duran bottle. Stable at 4°C for >2 years.

(**i**) *D-Glucose/glycerol LC standard*.—10 mg/mL of each containing sodium azide (0.02%, w/v). Stable for >4 years at 4°C.

(**j**) *Sodium maleate buffer*.—50 mM, pH 6.0 plus 2 mM $CaCl_2$ and 0.02% sodium azide. Dissolve 11.6 g maleic acid in 1600 mL deionized water and adjust the pH to 6.0 with 4 M (160 g/L) NaOH solution. Add 0.6 g calcium chloride ($CaCl_2.2H_2O$) and adjust the volume to 2 L. Stable for approximately 2 weeks at 4°C.

(**k**) *MES buffer*.—This can be used as an alternative to sodium maleate buffer; **C(j)**, 50 mM, pH 6.0 plus 2 mM $CaCl_2$. Dissolve 19.5 g MES [2-(*N*-morpholino) ethanesulfonic acid] in 1600 mL deionized water, and adjust the pH to 6.0 with 4 M (160 g/L) NaOH solution. Add 0.6 g calcium chloride ($CaCl_2.2H_2O$) and adjust the volume to 2 L. Solution is stable for approximately 2 weeks at 4°C.

(**l**) *Tris Base, 0.75 M*.—Add 90.8 g Tris base to approximately 800 mL distilled water and dissolve. Adjust to pH 11.0. Adjust volume to 1 L. Stable for >1 year at room temperature.

(**m**) *Acetic acid solution, 2 M*.— Add 115 mL glacial acetic acid (Fluka 45731; Sigma-Aldrich Ireland Ltd) to a 1 L volumetric flask. Dilute to 1 L with distilled water. Stable for >1 year at room temperature.

(**n**) *Sodium azide solution (0.02%, w/v)*.—Add 0.2 g sodium azide to 1 L deionized water and dissolve by stirring. Stable at room temperature for >1 year.

(**o**) *Cleaning solution*.—Micro (International Products Corp., Trenton, NJ). Make a 2% solution with deionized water.

(**p**) *pH standards*.—Buffer solutions at pH 4.0, 7.0, and 10.0.

(**q**) *Deionized water.*

(**r**) *Celite*.—Acid-washed, preashed.

(**s**) *Amberlite FPA53 (OH^-) resin, ion exchange capacity 1.6 meq/mL (minimum) and Ambersep 200 (H^+) resin ion exchange capacity 1.6 meq/mL (minimum).*

Items **(d)** and **(f)–(i)** are supplied in the rapid integrated TDF kit (Cat. No. K-RINTDF; Megazyme, Bray, Ireland), but preparations of reagents and buffers that meet the criteria as specified in the method above may also be used.

D. Preparation of Test Samples

Collect and prepare samples as intended to be eaten. Defat if >10% fat. For high-moisture samples, it may be desirable to freeze dry. Grind ca 50 g in a grinding mill, **B(a)**, to pass a 0.5 mm sieve. Transfer all material to a wide-mouthed plastic jar and mix well by shaking and inversion. Store in the presence of a desiccant.

E. Enzyme Purity

To ensure absence of undesirable enzymatic activities and effectiveness of desirable enzymatic activities, run standards listed in Table **991.43B** each time enzyme lot changes or at a maximum 6 month interval.

F. Enzymatic Digestion of Sample

(**a**) *Blanks*.—With each set of assays, run two blanks along with samples to measure any contribution from reagents to residue.

(**b**) *Samples.—(1) Weigh duplicate 1.000 ± 0.005 g samples accurately into 250 mL polypropylene bottles.*

(*2*) *Wet the sample with 1.0 mL ethanol (or IMS)*.—Add 35 mL of 50 mM sodium maleate buffer, **C(j)**, or MES buffer, **C(k)**, and a 7 × 30 mm stirrer bar to each bottle. Place bottles on a 2mag Mixdrive 15 magnetic stirrer apparatus in a water bath set at 37°C, **B(g)**. Stir the contents at 170 rpm for 10 min to equilibrate to 37°C. Alternatively, transfer the bottles (without stirrer bar) to a Grant OLS 200 shaking incubation bath, **B(g)**, (or similar), secure in place with the shaker frame springs, or a polypropylene holder (Figure **2017.16B**), and shake at 150 rpm in orbital motion for 10 min.

(*3*) *Incubation with pancreatic α-amylase plus AMG*.—Add 5.0 mL PAA/AMG solution, **C(e)**, (PAA 4 KU/5 mL and AMG 1.7 KU/5 mL) to each bottle, cap the bottles, and incubate the reaction solutions at 37°C with stirring at 170 rpm for exactly 4 h using a magnetic stirrer bar and a 2mag Mixdrive 15 magnetic stirrer apparatus; alternatively incubate in a shaking water bath maintained at 37°C at 150 revolutions/min (orbital motion) for exactly 4 h. Alternatively, if employing the ammonium sulphate suspension of PAA/AMG [PAA (2 KU/mL)/AMG (0.85 KU/mL); *see* **C(e)**, alternative], gently swirl the suspension before use and add 2.0 mL of this suspension and 3 mL maleate buffer, **C(j)**, or MES buffer, **C(k)**, to each bottle and incubate as indicated.

(*4*) *Adjustment of pH to approximately 8.2 (pH 7.9–8.4), Inactivation of α-amylase and AMG*.—After 4 h, remove all sample bottles from the stirring or shaking water bath, and immediately add 3.0 mL of 0.75 M Tris base solution, **C(l)**, to adjust pH to approximately 8.2 (7.9–8.4), at which pH AMG has no activity. Immediately, slightly loosen the caps of the sample bottles, place the bottles in a boiling water bath (nonshaking; 95–100°C), and incubate for 20 min with occasional agitation (by hand). This inactivates both PAA and AMG. With a thermometer, ensure that the final temperature of the bottle contents is >90°C. Checking just one bottle is adequate.

(At the same time, if only one shaker bath is available, increase the temperature of the shaking incubation bath to 60°C in readiness for the protease incubation step).

(*5*) *Cool and protease treatment*.—Remove all sample bottles from the hot water bath and cool to approx. 60°C. Add 0.1 mL protease suspension, **C(f)**, with a positive displacement dispenser (solution is thick) and incubate at 60°C for 30 min.

(*6*) *pH adjustment*.—Add 4.0 mL of 2 M acetic acid, **C(m)**, to each bottle and mix. This gives a final pH of approximately 4.3.

(7) Add *internal standard*.—To each sample, add 1 mL of 100 mg/mL glycerol (or diethyleneglycol) internal standard solution **C(g)**.

(*8*) *Proceed to step **G(a)***.

G. Determination of IDF + SDFP

(**a**) *Precipitation of SDFP and recovery of IDF + SDFP*.—To each sample, add 207 mL (measured at room temperature) of 95% (v/v) EtOH or IMS preheated to 60°C and mix thoroughly. Allow the precipitate to form at room temperature for 60 min (overnight precipitation is acceptable).

(**b**) *Filtration setup*.—Tare crucible containing Celite to nearest 0.1 mg. Wet and redistribute the bed of Celite in the crucible, using 15 mL of 78% (v/v) EtOH (or IMS) from wash bottle. Apply suction to crucible to draw Celite onto fritted glass as an even mat. Discard these washings.

(**c**) *Filtration*.—Using vacuum, filter precipitated enzyme digest, **G(a)**, through crucible. Using a wash bottle with 78%, v/v EtOH or IMS, quantitatively transfer all remaining particles to crucible and wash the residue successively with two 15 mL portions of 78%, v/v EtOH or IMS. Retain filtrate and washings for determination of SDFS, **H(a)**.

(**d**) *Wash*.—Transfer the crucible to a "waste" buchner flask and, using a vacuum, wash residue successively with two 15 mL portions of 95% (v/v) EtOH or IMS and then acetone. Discard these washings. Draw air through the crucibles for at least 2 min to ensure all acetone is removed before drying crucibles in an oven.

(**e**) *Dry crucibles*.—Loosely cover the crucibles with aluminium foil to prevent sample loss, and then dry the crucibles containing residue overnight in a 103°C oven.

(**f**) *Cool crucible*.—Cool crucible in desiccators for approximately 1 h. Weigh crucible containing dietary fiber residue and Celite to nearest 0.1 mg. To obtain residue weight, subtract tare weight, i.e., weight of dried crucible and Celite.

(**g**) *Protein and ash determination*.—The residue from one crucible is analyzed for protein, and the second residue of the duplicate is analyzed for ash. Perform protein analysis on residue using Kjeldahl or combustion methods. Use 6.25 factor for all cases to calculate g of protein. For ash analysis, incinerate the second residue for 5 h at 525°C. Cool in desiccator and weigh to nearest 0.1 mg. Subtract crucible and Celite weight to determine ash.

(**h**) *Proceed to step **I(a)***.

H. Determination of SDFS

Proper deionization of the filtrate is an essential part of obtaining quality chromatographic data on SDFS. Refer to Figure **2017.16E** to see patterns of glycerol and D-glucose in the presence and absence of buffer salts. To ensure that the resins being used are of adequate deionizing capacity, add 0.1 mL protease suspension, **C(f)**, to 40 mL either maleate buffer, **C(j)**, or MES buffer, **C(k)**, along with 3.0 mL of 0.75 M Tris base solution, **C(l)**, 4.0 mL of 2 M acetic acid, **C(m)**, 1 mL glycerol internal standard (100 mg/mL), **C(g)**, and 1 mL D-glucose solution (100 mg/mL). Concentrate this solution to dryness on a rotary evaporator and redissolve the residue in 32 mL deionized water. To 5 mL of this solution in a 13 mL polypropylene tube, **B(s)**, add 1.5 g Amberlite FPA53 (OH^-) resin, **C(s)**, and 1.5 g Ambersep 200 (H^+) resin, **C(s)**, and swirl the contents regularly over 5 min. Allow the resin to settle and remove the supernatant (1.5–2.0 mL) with a syringe, **B(cc)**, and filter through a polyvinylidene fluoride filter, pore size 0.45 µm, **B(z)**. Inject an aliquot (50 µL) of this solution onto the TSKgel G2500PW_{XL} columns [Bio-Rad deionization precartridges, **B(v)**, in place]. No salt peaks should be seen on HPLC.

(**a**) *Filtrate recovery, deionization, and LC analysis*.—Set aside the filtrate from one of the sample duplicates, **G(c)**, to use in case of spills or if duplicate SDFS data are desired. Transfer the filtrate from the second sample replicate, **G(c)**, into a 500 mL measuring cylinder. Adjust the volume to 300 mL with 78% (v/v) aqueous ethanol, **C(b)**, transfer to a 1 L beaker, and mix thoroughly. Transfer approximately 75 mL (approximately 25%) of this solution to a 500 mL evaporator flask and concentrate with a rotary evaporator to dryness at 50°C. (*Note*: it is not essential to quantitatively transfer all solution because SDFS is determined by the ratio of these peaks on HPLC to that of glycerol internal standard).

(**b**) *Deionization of sample*.—Dissolve the residue in the evaporator flask in 8 mL deionized water and transfer 5 mL of this solution to a 13 mL polypropylene tube, **B(s)**, containing 1.5 g Amberlite FPA53 (OH^-) resin and 1.5 g Ambersep 200 (H^+; Figure **2017.16F**). Cap the container and invert the contents regularly over 5 min. Alternatively, if the ammonium sulphate suspension of PAA/AMG is used for starch digestion [*see* **C(e)**, alternative], then use 2 g Amberlite FPA53 (OH^-) resin and 2 g Ambersep 200 (H^+) to ensure effective removal of most of the ions in the sample.

(**c**) *Prepare samples for LC analysis*.—Remove a sample (approximately 1.5–2.0 mL) of the supernatant solution from the resin slurry (Figure **2017.16F**) with a syringe, **B(cc)**, and filter through a polyvinylidene fluoride filter, pore size 0.45 µm, **B(z)**. Use this solution as the sample extract for step **H(f)**. HPLC patterns for nondeionized sample, sample deionized with resin in tube, and sample of desalted preparation run onto TSKgel G2500PW_{XL} columns through Bio-Rad deionization precartridges are shown in Figure **2017.16E**.

(**d**) *Determine the response factor for D-glucose*.—Because D-glucose provides an LC RI response equivalent to the response factor for the nondigestible oligosaccharides that make up SDFS, D-glucose is used to calibrate the LC and the response factor is used for determining the mass of SDFS. Use a 100 µL LC syringe, **B(dd)**, to fill the 50 µL injection loop with the D-glucose/glycerol internal standard solution, **C(i)**. Inject in duplicate. Calculate the response factor according to **I(b)(1).**

(**e**) *Calibrate the area of the chromatogram to be measured for SDFS*.—Use a 100 µL LC syringe, **B(dd)**, to fill the 50 µL injection loop with retention time standard, **C(h)**. Inject in duplicate. Determine the demarcation point between DP2 and DP3 oligosaccharides (disaccharide maltose versus higher oligosaccharides; Figure **2017.16D**).

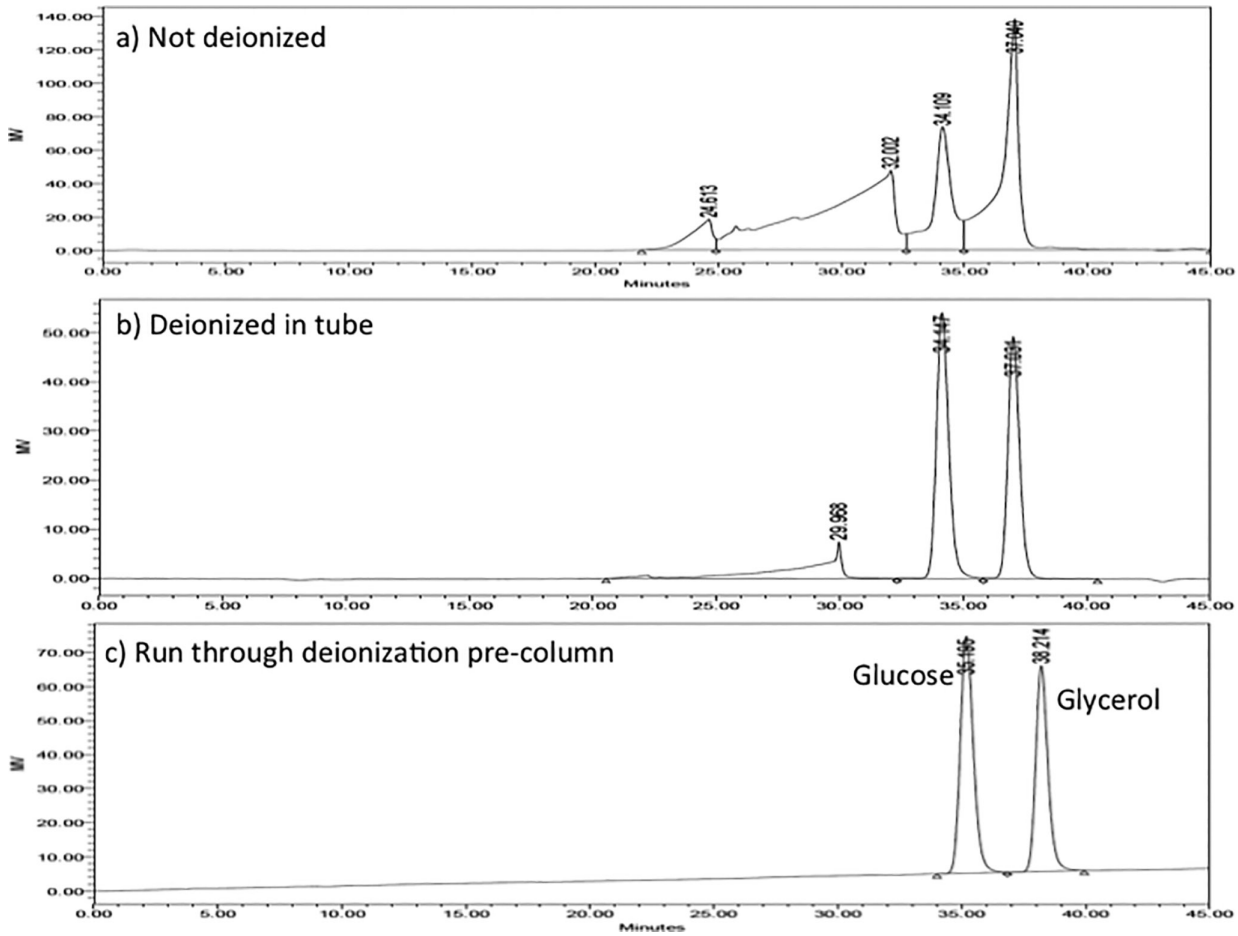

Figure 2017.16E. Chromatograms on TSKgel G2500PWXL columns of glucose/glycerol mixtures. A mixture of glycerol (100 mg) and glucose (100 mg) was analyzed according to the RINTDF procedure. The ethanolic filtrate (for SDFS determination) was concentrated to dryness and redissolved in 32 mL deionized water. A sample of this was analyzed by HPLC (a) directly with no deionization and no Bio-Rad deionization precartridges in place; (b) a sample (5 mL) was deionized by mixing with 1.5 g Amberlite FPA53 (OH^-) and 1.5 g Ambersep 200 (H^+) resins over 5 min and the supernatant was analyzed by HPLC with no Bio-Rad deionization precartridges in place; and (c) Sample (b) was analyzed with a Bio-Rad deionization precartridges in place. Deionization with resins in a polypropylene tube, as described here, removes >95% of the salt from the sample, thus ensuring more efficient use of the expensive Bio-Rad deionization precartridges. This deionization step increases the effectiveness of the deionization cartridges and allows up to 10 times more samples to be chromatographed before the need to regenerate or replace the deionization cartridges.

(**f**) *Determine peak areas of SDFS (PA_{SDFS}) and internal standard (PA_{IS}) in chromatograms of sample extracts*.—Inject sample extracts, **H(c)**, on LC. Record areas of all peaks of DP greater than the DP2/DP3 demarcation point as PA_{SDFS}. Record the peak area of internal standard as PA_{IS}.

(**g**) *Proceed to step **I(b)***.

I. Calculations for Total Dietary Fiber as IDF + SDFP + SDFS

(a) *IDF + SDFP (by gravimetry)*.—Blank (B, mg) determination.

$$B = \frac{BR_1 + BR_2}{2} - P_B - P_A$$

where BR_1 and BR_2 = residue mass, in mg, for duplicate blank determinations, respectively; and P_B and P_A = mass, in mg, of protein and ash, respectively, determined on first and second blank residues.

$$[IDF + SDFP]\,mg/100\,g = \frac{[(R_1 + R_2)/2 - P_B - P_A - B]}{(M_1 + M_2)/2} \times 100$$

$$[IDF + SDFP]\,g/100\,g = \frac{[IDF + SDFP]\,mg/100g}{1{,}000}$$

where R_1 = residue mass 1 from M_1 in mg; R_2 = residue mass 2 from M_2 in mg; M_1 = test portion mass 1 in g; M_2 = test portion mass 2 in g; P_A = ash mass in mg from R_1; and P_B = protein mass in mg from R_2.

(**b**) *SDFS (by HPLC)*.—(*1*) *Determination of* D*-glucose response factor*.—Obtain the values for the peak areas of D-glucose and internal standard (glycerol) from duplicate chromatograms. The ratio of peak area of D-glucose/peak area of glycerol to the ratio of the mass of D-glucose/mass of glycerol is the "response factor." The average response factor for D-glucose is approximately 0.82 versus glycerol.

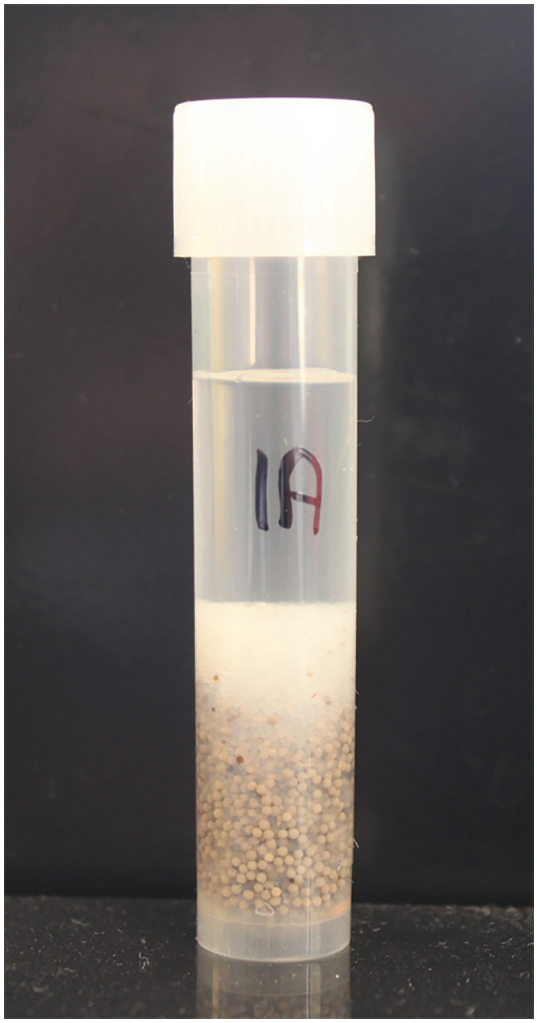

Figure 2017.16F. Deionization of samples for HPLC. Five milliliters concentrated eluate mixed with 1.5 g Amberlite FPA53 (OH^-) and 1.5 g Ambersep 200 (H^+) resins in a polypropylene tube.

$$\text{Response factor (Rf)} = \frac{PA_{IS}}{PA_{Glu}} \times \frac{Wt_{Glu}}{Wt_{IS}}$$

where PA_{Glu} = peak area of D-glucose; PA_{IS} = peak area of internal standard (glycerol); Wt_{Glu} = mass of D-glucose in standard; and Wt_{IS} = mass of internal standard (glycerol) in standard.

(*2*) *Determination of SDFS.*—

$$SDFS(mg/100\,g) = \frac{Rf \times Wt_{IS} \times PA_{SDFS}}{PA_{IS}} \times \frac{100}{M}$$

$$SDFS(g/100\,g) = \frac{SDFS(mg/100\,g)}{1{,}000}$$

where Rf = the response factor; Wt_{IS} = mg of internal standard contained in 1 mL of glycerol internal standard solution (100 mg/mL; i.e., 100 mg) pipetted into sample before filtration; PA_{SDFS} = the peak area of the SDFS; PA_{IS} = the peak area of the glycerol internal standard; M = the test portion mass, M_1 or M_2 of the sample whose filtrate was concentrated and analyzed by LC.

(**c**) *Total dietary fiber.*—

$$\text{Total dietary fiber (g/100g)} = [IDF + SDFP]\ (g/100g) + SDFS\ (g/100g)$$

Calculations can be simplified by using an Excel-based calculator (Supplementary Information; RINTDF Mega-Calc, https://secure.megazyme.com/Rapid-Integrated-Total-Dietary-Fiber-Assay-Kit).

Results and Discussion

To simulate food digestion in the small intestine, a combination of gentle shaking or stirring in the presence of PAA/AMG digestion at 37°C is used in both AOAC **2009.01** and the new RINTDF method. In the RINTDF method, the incubation time with PAA/AMG mixture is reduced to 4 h, to better simulate the likely time of residence of food in the small intestine (6, 7). To ensure that results obtained for samples containing resistant starches (RS_2, RS_3, and RS_4) using a 4 h incubation are in agreement with known values from ileostomy studies of samples and ingredients rich in these starches, the concentrations of both PAA and AMG were increased to levels (PAA, 4 KU/test and AMG, 1.7 KU/test) above which further increases in activity (as much as 4-fold) gave no increase in the levels of nonresistant starch, nor decrease in the levels of measured resistant starch over a wide range of starch-containing samples (6).

Raw data for the dietary fiber collaborative study are shown in Table 3, with Cochran and Grubbs outliers indicated. The statistical results, after removal of the outliers and data from laboratories 2 and 12, are shown in Table 4. As previously stated, the samples used for this collaborative study were chosen to be challenging, i.e., with an emphasis on analyzing products containing resistant starch and nondigestible oligosaccharides. As can be seen from Table 4, the s_r for TDF ranged from 0.27 to 0.76 g/100 g, and the s_R ranged from 0.54 to 3.99 g/100 g. When compared with statistical results for previously adopted dietary fiber methods (Table 2), the level and range of variability for the current method were similar to those of the other dietary fiber methods, most likely influenced in all cases by the significant number of technique-dependent manual operations (15). Repeatability, reproducibility, and HorRat were within the range of performance characteristics typically found for dietary fiber methods. In previously adopted methods, the s_R ranged from 0.04 to 9.49 g/100 g and the RSD_R from 1.58 to 66.25% (Table 2). As stated, data from laboratories 2 and 12 were removed from the statistical evaluation of the method as summarized in Table 4 because the final operation in measurement of SDFS by HPLC was performed in the method author's laboratory (laboratories 2 and 12 did not have the required HPLC columns). However, the statistical evaluation of data from all 13 laboratories was also performed and is shown in Table 5. Clearly, the statistical parameters are little changed from those obtained on evaluation of the data from the 11 laboratories.

It is essential to ensure that the increased levels of enzyme, especially the AMG, do not lead to hydrolysis of other dietary fiber components such as FOS, galacto-oligosaccharides, resistant maltodextrins, etc. Studies confirming this were previously reported (6, 8). After incubation of the sample with PAA/AMG, the pH of the incubation mixture is increased to approximately 8.2. This prevents enzymatic hydrolysis of that component of resistant starch that is solubilized during the subsequent heating process. Heating of the sample solutions to approximately

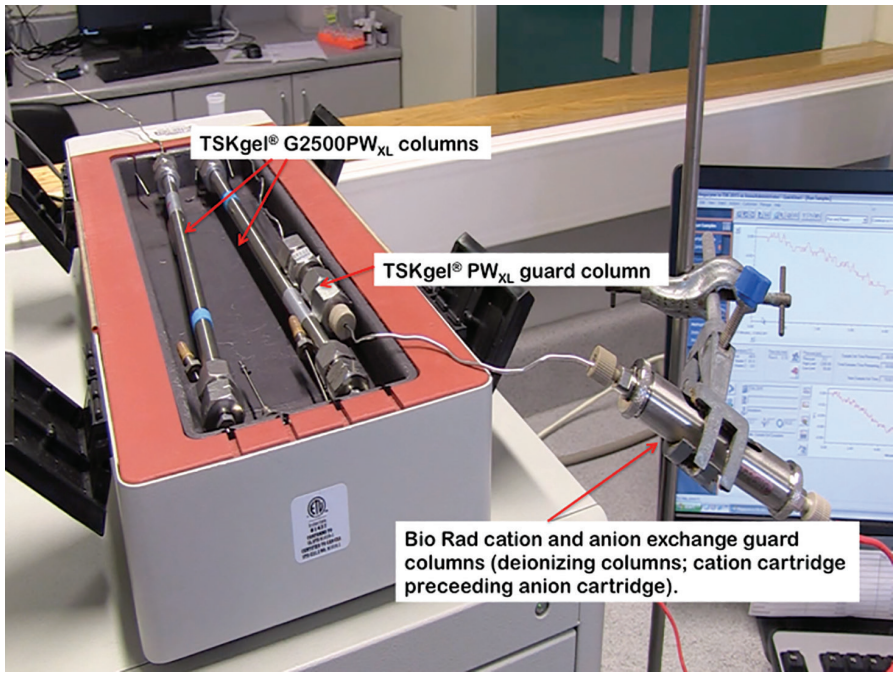

Figure 2017.16G. Photograph of HPLC setup for measurement of SDFS, highlighting the two TSKgel G2500PWXL columns, the TSKgel PWXL guard column and a Bio-Rad cartridge containing cation and anion deionization cartridges.

100°C inactivates the PAA and AMG and promotes denaturation of protein, which is essential for efficient protein hydrolysis by protease. IDF + SDFP is recovered gravimetrically after alcohol precipitation of the SDFP, and combining this result with SDFS determined by HPLC gives the value for TDF.

Reducing the incubation time with PAA/AMG from 16 to 4 h has the advantage of removing the risk of microbial contamination of the sample during extended incubation. Thus, sodium azide is not required in the incubation buffer. However, inclusion of sodium azide in the maltodextrin LC retention time standard solution and the glucose/glycerol LC standard solution is recommended as this stabilizes these solutions for several years.

In this method, sample concentrates containing SDFS were analyzed using TSKgel G2500PW$_{XL}$ columns with in-line removal of anions and cations (**Figure 2017.16G**; 12). The in-line deionization cartridges obtained from Bio-Rad have a limited capacity, being able to deionize just 25–30 samples. To reduce the very significant cost factor in using these cartridges, SDFS concentrates are first deionized in a polypropylene tube containing anion and cation exchange resins prior to HPLC. This predeionization removes 90–95% of ions from the SDFS and extends the life of the Bio-Rad HPLC deionization cartridges 10–20-fold.

In the current method, SDFS is analyzed on TSKgel G2500PW$_{XL}$ columns with a glycerol internal standard (6). If the sample being analyzed contains glycerol, diethylene glycol is a suitable alternative internal standard. In AOAC **2009.01**, a Waters Sugar-Pak column is employed with D-sorbitol as the internal standard. However, on the Sugar-Pak column, inulinotriose, a significant component of hydrolyzed fructan, elutes at the same point as disaccharides and thus is not measured as dietary fiber. Complete separation of all trisaccharides is obtained using TSKgel G2500PW$_{XL}$ columns.

Based on the HPLC chromatographic traces supplied by collaborating laboratories along with their results, it was evident that in some laboratories the HPLC systems were not run optimally, as evidenced by a significant upward slant of the baseline of the chromatogram during a run. This pattern indicates that the column is partially blocked and operating pressure is likely above the recommended level. Backwashing of the column over 24–48 h prior to its continued use will reoptimize the column performance.

Conclusions

AOAC **2009.01** is recognized by the Codex Alimentarius, the U.S. Food and Drug Administration, and food authorities worldwide as the reference method for measuring TDF in foods and food ingredients. AOAC **2017.16** was developed and validated to address problems that have been identified over the past 8 years when applying AOAC **2009.01** to the measurement of fiber in specific fiber ingredients. This has been achieved by modifying incubation conditions with PAA/AMG to more closely simulate physiological conditions (pH 6.0, 37°C, 4 h), simplifying sample preparation and HPLC (for SDFS determination), and removing or minimizing the use of hazardous chemicals in the method. AOAC **2017.16** gives accurate and quantitative measurements of dietary fiber in all foods and food ingredients.

AOAC **2017.16**, like **2009.01**, measures TDF as a summation of higher molecular weight dietary fiber (IDF + SDFP; by gravimetry) and SDFS (by HPLC). AOAC **2011.25**, a modification of AOAC **2009.01**, allows for separate measurements of IDF and SDF (SDFP + SDFS). A similar modification of AOAC **2017.16** has been developed within Megazyme but awaits either single-laboratory validation or multilaboratory validation through AOAC INTERNATIONAL.

Collaborator Comments

No negative comments were received concerning the method, but one collaborator did notice an allergic reaction to the PAA/AMG powder mixture. In response to this, the assay protocol now includes an option in which a nonallergic analyst can suspend the enzyme powder mixture in ammonium sulphate solution to produce a stabilized liquid form that reduces the risk to a susceptible analyst. One collaborator did not have access to the deionizing precolumns, so they deionized the samples according to the formats in AOAC **2001.03** (11). The same collaborator did not have a water bath with orbital motion and was thus advised to position the incubation containers at an angle of approximately 45° parallel to the direction of shaking to ensure that the sample did not settle to the bottom of the container during incubation. One collaborator asked for further advice on where to distinguish between SDFS and disaccharides on the HPLC pattern.

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Hayfa Salman, Australian Export Grains Innovation Centre (North Ryde, Australia)

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