

AOAC Official Method 2011.25
Insoluble, Soluble, and Total Dietary Fiber in Foods
Enzymatic-Gravimetric-Liquid Chromatography
First Action 2011

[Applicable to plant material, foods, and food ingredients consistent with CODEX Alimentarius Commission Definition adopted in 2009 and modified slightly in 2010 (ALINORM 09/32/REP and ALINORM 10/33/REP, respectively), including naturally occurring, isolated, modified, and synthetic polymers meeting that definition.]

See Tables 2011.25A–H for the results of the interlaboratory study supporting acceptance of the method.

A. Principle

A method is described for the measurement of insoluble, soluble, and total dietary fiber (IDF, SDF, and TDF, respectively), inclusive of the resistant starch (RS) and the water:alcohol soluble nondigestible oligosaccharides and polysaccharides of DP ≥ 3 . The method combines the key attributes of AOAC *Official Methods of Analysis*SM 985.29 (and its extensions, 991.42 and 993.19), 991.43, 2001.03, and 2002.02. Duplicate test portions are incubated with pancreatic α -amylase and amyloglucosidase (AMG) for 16 h at 37°C in sealed 250 mL bottles while mixing with sufficient vigor to maintain continuous suspension. During this step, 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 and temporary heating. Protein in the sample is then digested with protease. For the measurement of IDF, the digestate is filtered and the IDF is determined gravimetrically after correction for any protein or ash in the residue. For the measurement of the water soluble, but water:alcohol insoluble dietary fiber (SDFP), ethanol is added to the filtrate of the IDF; the precipitated SDFP is captured by filtration and determined gravimetrically after correction for any protein or ash in the precipitate. Nonprecipitable, water:alcohol soluble dietary fiber (SDFS) in the filtrate is recovered by concentrating the filtrate, deionizing through ion exchange resins, concentrating, and quantitating by LC, or, alternatively, by concentrating the filtrate and simultaneously deionizing and quantitating by LC.

B. Apparatus

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

(b) *Digestion bottles*.—250 mL Fisherbrand® soda glass, wide-mouthed bottles with polyvinyl-lined cap (Cat. No. FB73219, equivalent: Cat. No. 02-911-916; Fisher USA, www.fisher.co.uk/

supplies/fisherbrand.html) or 250 mL polypropylene bottles with polypropylene caps.

(c) *Fritted crucible*.—Gooch, fritted disk, Pyrex® 50 mL, pore size, coarse, ASTM 40–60 μm (Corning No. 32940-50C® or equivalent; <http://www.labplanet.com/corning-crucible-gooch-high-c-50-ml-32940-50c.html>). 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% cleaning solution, C(i), 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 h and record mass 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, large-capacity (20–24 L) with covers; capable of maintaining temperature of $37 \pm 1^\circ\text{C}$ and $60 \pm 1^\circ\text{C}$; equipped with automatic timers for on-off operation or equivalent (e.g., Grant® OLS 200 shaking incubation bath; <http://www.bioresearchonline.com/product.mvc/Grant-OLS200-Orbital-Shaking-Water-Bath-0001>). Ensure that shaking action/sample agitation in water bath used is sufficient to maintain sample solids in suspension and no residue build-up or rings of sample material form in the digestion bottle during the enzymatic digestions. A linear motion (back and forth shaker) can be used if the bottles are placed at 45° to ensure adequate agitation (if the bottles are vertical or horizontal there will not be sufficient agitation to ensure that the sample remains suspended). Alternatively, a 2 mag Mixdrive 15 submersible magnetic stirrer apparatus (http://www.2mag.de/english/stirrer/multiple/stirrer_multiple_04_mixdrive6_15.html) can be used in a water bath maintained at $37 \pm 1^\circ\text{C}$ with a Julabo circulating heater (<http://www.julabo.de/>). Samples are stirred in digestion bottles, (b), with a 7×30 mm stir bar at ~ 170 rpm (Figure 2011.25A).

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

(i) *Ovens*.—Two, mechanical convection, set at $105 \pm 2^\circ\text{C}$ and $130 \pm 3^\circ\text{C}$, respectively.

(j) *Timer*.

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

(l) *pH meter*.

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

Table 2011.25A. Interlaboratory study results for IDF

Sample/parameter	A	B	C	D	E	F	G	H
No. of labs	14	15	15	15	13	13	12	13
Mean, %	25.74	4.79	9.23	1.05	8.26	11.63	11.52	9.90
S_r	0.52	0.18	0.71	0.13	0.19	0.48	0.33	0.29
S_R	1.23	0.47	2.24	0.42	0.48	1.12	1.21	0.77
RSD_r	2.03	3.76	7.67	12.25	2.26	4.11	2.87	2.98
RSD_R	4.76	9.82	24.35	39.64	5.81	9.65	10.53	7.80
HorRat	1.94	3.11	8.51	9.98	2.00	3.49	3.80	2.75

Table 2011.25B. Interlaboratory study results data for SDF

Sample/parameter	A	B	C	D	E	F	G	H
No. of labs	14	14	15	15	14	13	15	13
Mean, %	3.80	5.58	17.32	10.74	12.94	4.56	11.29	8.30
S _r	0.44	0.28	1.03	0.46	0.50	0.83	0.71	0.51
S _R	0.99	0.85	1.30	1.12	1.52	1.66	1.61	1.33
RSD _r	11.71	5.10	5.97	4.33	3.86	18.10	6.29	6.18
RSD _R	26.20	15.20	7.53	10.46	11.73	36.40	14.25	16.07
HorRat	8.01	4.92	2.89	3.74	4.31	11.44	5.13	5.53

(n) *Dispensers*.—(1) 15 ± 0.5 mL for 78% EtOH or industrial methylated spirits (IMS), 95% ethanol or IMS, and acetone. (2) 40 ± 0.5 mL for buffer.

(o) *Cylinder*.—Graduated, 500 mL.

(p) *Magnetic stirrers and stirring bars*.

(q) *Rubber spatulas*.

(r) *Muffle furnace*.—525 ± 5°C.

(s) *Polypropylene columns*.—(If manual deionization is carried out.) For the interlaboratory study, Alltech SPE cartridges [Extract-Clean™ reservoirs (15 mL), Cat. No. 210315; http://www.discoverysciences.com/uploadedFiles/Home/SPE_Filtration_ExtractNUltraClean_p302to306.pdf] were used. Ten grams of cation or anion exchange resins were added to each column. The cartridges were connected with an Alltech Syringe Adapter™ (Cat. No. 210705), with cation exchange resin [Ambersep 200C (H⁺)] always at the top. Flow was controlled by pump or with an Alltech One-Way Stopcock (Cat. No. 211524; Figure 2011.25B). A method for resin regeneration was provided, but reuse of resins is no longer recommended since resin cost is minimal. However, Extract-Clean reservoirs and Syringe Adapters subsequently received were unsatisfactory. There was some leakage of liquid due to incomplete sealing. Subsequently, sample deionization has been achieved using a mixed bed resin of approximately 4 g cation [Ambersep 200C (H⁺)] and 4 g anion [Amberlite FPA53 (OH⁻)] in a Bio-Rad Laboratories Econo-Pac™ disposable chromatography column (Cat. No. 732-1010; http://www.bio-rad.com/webroot/web/pdf/psd/literature/Bulletin_9103.pdf) with an Alltech One-Way Stopcock (Cat. No. 211524; Figure 2011.25B).

(t) *Cation and anion exchange guard columns*.—(If online simultaneous deionization and quantitation are carried out.) Cation and anion exchange guard columns, H⁺ and CO₃³⁻ forms, respectively (Bio-Rad Laboratories, Cat. No. 125-0118, includes one cation and one anion cartridge), with guard column holder (Bio-Rad Laboratories, Cat. No. 125-0139) to hold the two guard

column cartridges in series, cation cartridge preceding anion cartridge.

(u) *Liquid chromatograph*.—(1) *Pump*.—Capable of precision metering at 0.5 mL/min at up to 10000 psi. Arrangement for HPLC of manually desalted samples on Waters Sugar-Pak® column (<http://www.waters.com/waters/support.htm?locale=?&cid=513789&lid=10069619>) is shown in Figure 2011.25C. [If using Sugar-Pak column and simultaneous on line deionization and quantitation, a second pump capable of precision metering at 0.05 mL/min at up to 10000 psi is needed to add disodium calcium ethylenediaminetetracetate (Na₂CaEDTA) via zero dead volume t-fitting to the line between the deionization cartridges and the quantitation column (see Figure 2011.25D).]

(2) *Filled loop injector*.—Equipped with 50 µL injection loop.

(3) *LC column*.—Waters Sugar-Pak 6.5 × 300 mm (Part No. WAT085188) or equivalent preceded by Waters Guard Pak LC precolumn inserts (Part No. WAT015209) or equivalent.

(4) *Refractive index (RI) detector*.—Capable of being maintained at 50°C.

(5) *Oven*.—To maintain a column temperature of 90°C.

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

(w) *Filters for disposable syringe*.—Polyvinylidene fluoride 0.45 µm, 13 or 25 mm.

(x) *Filters for water*.—Polyvinylidene fluoride, 0.45 µm, 47 mm.

(y) *Filter apparatus*.—To hold 47 mm, 0.45 µm filter, (x); to filter larger volumes of water.

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

(aa) *Syringes*.—Hamilton® 100 µL, 710SNR syringe.

(bb) *Rotary evaporator*.—Heidolph Laborota® 4000 or equivalent (http://www.lasallescientific.com/products/heidolph/BAL_LARO4000_Nov06_en1.pdf).

(cc) *Thermometer*.—Capable of measuring to 110°C.

(dd) *Freeze drying apparatus*.

Table 2011.25C. Interlaboratory study results for TDF

Sample/parameter	A	B	C	D	E	F	G	H
No. of labs	15	15	15	15	14	13	14	13
Mean, %	29.90	10.45	26.55	11.79	21.37	16.39	23.71	18.40
S _r	0.86	0.47	1.39	0.49	0.52	1.41	0.87	0.64
S _R	2.05	0.95	2.74	1.10	1.72	2.37	3.14	1.56
RSD _r	2.88	4.51	5.25	4.17	2.43	8.60	3.65	3.47
RSD _R	6.85	9.11	10.31	9.30	8.04	14.48	13.23	8.47
HorRat	2.85	3.24	4.22	3.37	3.19	5.51	5.33	3.28

Table 2011.25D. Interlaboratory study results for TDF measured directly

Sample/parameter	A	B	C	D	E	F	G	H
No. of labs	9	8	9	9	9	7	9	9
Mean, %	28.08	9.97	24.60	11.00	21.21	16.47	21.27	16.47
S _r	1.26	0.41	0.70	0.39	1.11	0.72	1.19	1.16
S _R	1.31	1.24	1.95	2.54	2.30	5.27	2.10	2.58
RSD _r	4.50	4.16	2.83	3.57	5.23	4.38	5.61	7.04
RSD _R	4.67	12.41	7.95	23.11	10.86	32.00	9.89	15.66
HorRat	1.93	4.39	3.22	8.29	4.30	12.20	3.92	5.97

C. Reagents

(a) *Ethanol, 95% (v/v) or alternatively use IMS.*—Specifications for preparation or commercial purchase: ethanol 84.83 (w%), 85.95 (v%); water 5.66 (w%), 4.52 (v%); 2-propanol 4.91 (w%), 5.00 (v%); methanol 4.60 (w%), 4.52 (v%). Alternatively, IMS can be prepared by mixing five volumes of 2-propanol with 95 volumes of denatured ethanol formula SDA-3A (SDA-3A alcohol consists of 100 volumes of 95% ethanol combined with five volumes of methanol).

(b) *Ethanol (or IMS), 78% (v/v).*—Place 207 mL water into 1 L volumetric flask. Dilute to volume with 95% ethanol or IMS. Mix.

(c) *Acetone.*—Reagent grade.

(d) *Stock AMG solution.*—3300 Units/mL in 50% (v/v) glycerol. Solution is viscous; for dispensing use positive displacement dispenser. AMG solution is stable for up to 5 years when stored at 4°C. (Note: One unit of enzyme activity is amount of enzyme required to release 1 micromole glucose from soluble starch per minute at 40°C and pH 4.5.) AMG solution should be essentially devoid of β-glucanase and β-xylanase and free of detectable levels of free glucose (see E).

(e) *Pancreatic α-amylase (50 Units/mL)/AMG (3.4 Units/mL).*—Immediately before use, dissolve 0.10 g purified porcine pancreatic α-amylase (~130 000 Units/g; Ceralpha method; AOAC Method 2002.01) in 290 mL sodium maleate buffer (50 mM, pH 6.0 plus 2 mM CaCl₂ and 0.02% sodium azide) and stir for 5 min. Add 0.3 mL AMG stock solution (see E).

(f) *Protease (50 mg/mL; ~350 Tyrosine Units/mL) in 50% (v/v) glycerol.*—Solution is viscous; for dispensing use positive displacement dispenser. Protease must be devoid of α-amylase and essentially devoid of β-glucanase and β-xylanase (see E). Use as supplied.

(g) *Deionized water.*

(h) *Celite.*—Acid-washed, pre-ashed (Megazyme G-CEL100

or G-CEL500, Megazyme International Ireland Ltd., Bray, County Wicklow, Ireland, <http://www.megazyme.com/Dynamic.aspx?control=CSViewProduct&categoryName=GeneralChemicals&productId=G-CEL100>; or Sigma-Aldrich Cat. No. C8656, http://www.sigmaaldrich.com/catalog/ProductDetail.do?D7=0&N5=SEARCH_CONCAT_PNO%7CBRAND_KEY&N4=C8656%7CSIGMA&N25=0&QS=ON&F=SPEC).

(i) *Cleaning solution.*—Micro-90® (International Products Corp., Burlington, NJ). Make a 2% solution (w/v) with deionized water.

(j) *Sodium maleate buffer.*—50 mM, pH 6.0 plus 2 mM CaCl₂ and 0.02% sodium azide. Dissolve 11.6 g maleic acid in 1600 mL distilled water and adjust pH to 6.0 with 4 M (160 g/L) NaOH solution. Add 0.6 g calcium chloride dihydrate and 0.4 g sodium azide [Note: Do not add the sodium azide until the pH has been adjusted. Acidification of sodium azide releases a poisonous gas. Handle sodium azide and maleic acid with caution only after reviewing MSDS, using appropriate personal protective gear and laboratory hood.] and adjust the volume to 2 L. Stable for >1 year at 4°C.

(k) *Trizma base.*—0.75 M (Sigma Cat. No. T-1503). Add 90.8 g Trizma base to approximately 800 mL distilled water and dissolve. Adjust volume to 1 L. Stable for >1 year at room temperature.

(l) *Acetic acid solution, 2 M.*—Add 115 mL glacial acetic acid (Fluka 45731) to a 1 L volumetric flask. Dilute to 1 L with distilled water.

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

(n) *Fructooligosaccharides.*—From chicory (Sigma Aldrich Cat. No. F8052).

(o) *LC retention time standard.*—Standard source having the distribution of oligosaccharides (DP ≥3) corn syrup solids (DE 16; Matsutani Chemical Industry Co., Ltd., Itami City, Hyogo, Japan; <http://www.matsutani.com/productsframeset.html>, maltodextrin TK-16; or Sigma Aldrich Cat. No. 419699), analyzed by LC.

Table 2011.25E. Interlaboratory study results for SDF as measured using online deionization

Sample/parameter	A	B	C	D	E	F	G	H
No. of labs	6	6	6	6	6	6	5	6
Mean, %	3.45	4.87	16.29	8.60	11.93	2.95	10.98	7.11
S _r	0.34	0.29	0.58	0.49	0.24	0.16	0.24	0.26
S _R	0.64	0.49	1.24	1.99	0.83	0.49	0.40	1.07
RSD _r	9.98	5.94	3.55	5.73	2.00	5.28	2.18	3.64
RSD _R	18.63	10.12	7.60	23.12	6.96	16.73	3.64	15.08
HorRat	5.61	3.21	2.89	7.99	2.53	4.92	1.31	5.07

Table 2011.25F. Interlaboratory study results for TDF (sum of IDF and SDF) as measured using online deionization

Sample/parameter	A	B	C	D	E	F	G	H
No. of labs	6	6	6	4	6	6	5	6
Mean, %	29.65	9.95	26.96	11.21	20.47	15.06	23.04	17.43
S _r	0.73	0.36	0.73	0.27	0.32	0.43	0.25	0.24
S _R	1.29	0.66	2.41	0.37	0.90	1.16	1.71	0.77
RSD _r	2.47	3.58	2.70	2.42	1.58	2.87	1.09	1.36
RSD _R	4.33	6.62	8.95	3.33	4.40	7.67	7.40	4.41
HorRat	1.81	2.34	3.67	1.20	1.73	2.88	2.97	1.69

Dissolve 2.0 g portion of oligosaccharide mixture and 0.50 g maltose in deionized water and transfer to 100 mL volumetric flask. [Alternatively, dissolve 2.5 g LC retention time standard mix from Integrated Total Dietary Fiber Assay Kit (K-INTDF; Megazyme International Ireland Ltd.), which contains maltodextrins with added maltose.] Pipet 10 mL of stock internal standard solution, (q). Bring to volume with 0.02% sodium azide solution, (u). Transfer solution to 100 mL polypropylene bottle. Stable at room temperature for 1 year.

(p) *Manual deionization.*—(1) *Original method.*—*Column 1.*—10 g Ambersep 200 (H⁺) resin, ion exchange capacity (R-H exchange capacity data supplied by manufacturer) 1.6 meq/mL (min), or equivalent (Rohm and Haas, France S.A.S.; see also Megazyme G-AMBH). *Column 2.*—10 g Amberlite FPA53 (OH⁻) resin, 1.6 meq/mL (min) or equivalent (Rohm and Haas, France S.A.S.; see also Megazyme G-AMBOH) packed in separate columns [Extract-Clean reservoirs (15 mL), B(s)] for analysis of each test portion. Before connecting the columns, wash the resin in each column with sufficient H₂O to obtain a pH value of 4–7 for column 1 and 7–8 for column 2.

(2) *Updated method.*—Mix approximately 4 g Ambersep 200 (H⁺) resin or equivalent, 1.6 meq/mL (min) or equivalent (Rohm and Haas, France S.A.S.; see also Megazyme G-AMBH) with approximately 4 g Amberlite FPA53 (OH⁻) resin, 1.6 meq/mL (min) or equivalent (Rohm and Haas, France S.A.S.; see also Megazyme G-AMBOH), ion exchange capacity (R-OH exchange capacity data supplied by manufacturer), in a 100 mL beaker and slurry with a minimum volume of distilled water. Pour this mixture into a Bio-Rad Econo-Pac disposable chromatography column (Cat. No. 732-1010) fitted with an Alltech One-Way Stopcock (Cat. No. 211524). Place a small wad of cotton wool into the top of the column and wash the resin with 20 mL distilled water. The column is then ready for use. If alternative resins are used, determine that carbohydrates are not retained by the resin by preparing a test

solution consisting of 1 mL of 100 mg/mL internal standard and 2.5 mL of 10 mg/mL fructooligosaccharides diluted to 10 mL. Add 2 mL of this solution to the mixed bed deionization resin (approximately 8 g) as per I(e). Recovery of the internal standards and fructooligosaccharides should match that of the solution injected directly onto the LC.

(q) *D-sorbitol (stock internal standard).*—100 mg/mL. [Note: Sorbitol is present in some foods and food products, such as plums (prunes), pears, apples, and Polydextrose® and may also be added to foods. If sorbitol is present, use of the internal standard will result in artificially low SDFS quantity. In these cases, the external standard method of calculation should be used. Addition of the internal standard is still recommended to use as a retention time reference.] Weigh 100 g dry D-sorbitol into a beaker, dissolve in water, transfer to a 1 L volumetric flask with water, and dilute to volume. Transfer to a polypropylene bottle and add 0.2 g sodium azide as a preservative. (Note: Handle sodium azide with caution only after reviewing MSDS, using appropriate personal protective gear and laboratory hood.) Seal well. Stable at room temperature for >1 year.

(r) *D-glucose LC standards (5, 10, 20 mg/mL).*—Accurately weigh 0.5, 1.0, and 2.0 g portions of high-purity (>99.5%) D-glucose (Sigma Chemical Co.; <http://www.sigmaldrich.com/ireland.html>) and transfer to three separate 100 mL volumetric flasks respectively. To each flask pipet 10 mL of internal standard, (q). Bring to volume with 0.02% sodium azide solution, (u). Transfer solutions to 100 mL polypropylene bottles. Stable at room temperature for 1 year.

(s) *Disodium calcium ethylenediaminetetraacetate (Na₂CaEDTA; 50 mg/L; if using Sugar-Pak LC column and manual deionization).*—Dissolve 50 mg Na₂CaEDTA in about 500 mL deionized water in a 1 L volumetric flask; dilute to volume.

(t) *Na₂CaEDTA (500 mg/L; if using Sugar-Pak LC column and simultaneous online deionization and quantitation).*—Dissolve

Table 2011.25G. Interlaboratory study results for SDF calculated using external standard approach

Sample/parameter	A	B	C	D	E	F	G	H
No. of labs	11	12	12	12	12	11	11	10
Mean, %	3.94	5.49	16.04	10.06	12.52	3.92	11.34	8.09
S _r	0.45	0.54	1.48	0.81	0.67	0.69	0.44	0.63
S _R	1.00	1.11	2.02	1.81	2.10	1.20	0.95	1.99
RSD _r	11.42	9.92	9.21	8.06	5.37	17.58	3.93	7.81
RSD _R	25.40	20.20	12.62	17.95	16.74	30.58	8.39	24.58
HorRat	7.80	6.53	4.79	6.35	6.12	9.39	3.02	8.42

Table 2011.25H. Interlaboratory study results for TDF calculated using external standard approach

Sample/parameter	A	B	C	D	E	F	G	D	H
No. of labs	12	12	12	12	12	10	12		10
Mean, %	30.05	10.35	25.52	11.20	20.93	15.58	23.52		18.05
S _r	1.05	0.59	1.52	0.85	0.96	0.85	0.77		0.60
S _R	1.95	1.13	2.08	1.71	1.97	1.27	2.61		1.55
RSD _r	3.49	5.67	5.95	7.57	4.61	5.49	3.26		3.32
RSD _R	6.47	10.90	8.13	15.26	9.41	8.18	11.08		8.61
HorRat	2.70	3.87	3.31	5.49	3.72	3.09	4.46		3.33

500 mg Na₂CaEDTA in about 500 mL deionized water in a 1 L volumetric flask; dilute to volume.

(u) *Sodium azide solution (0.02%, w/v)*.—Add 0.2 g sodium azide to 1 L deionized water and dissolve by stirring. (Note: Handle sodium azide with caution only after reviewing MSDS, using appropriate personal protective gear and laboratory hood.) Stable at room temperature for >1 year.

Items (d)–(f) and (n) are supplied in K-INTDF (Megazyme International Ireland Ltd), but preparations of reagents and buffers which 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, i.e., baking mixes should be prepared and baked, pasta should be cooked, etc. Defat per AOAC 985.29 if >10% fat. For high-moisture samples (>25%), 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, seal, and mix well by shaking and inversion. Store jar 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 985.29 each time enzyme lot changes or at a maximum 6 month interval.

F. Enzymatic Digestion of Sample

(a) *Blanks*.—With each assay, run 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 Fisherbrand bottles, B(b).

(2) *Addition of enzymes*.—Wet sample with 1.0 mL ethanol (or IMS) and add 40 mL pancreatic α-amylase/AMG mixture, C(e), to each bottle. Cap the bottles. Transfer bottles to the orbital or linear shaking incubation bath and secure bottles in place with the springs in the shaker frame. Alternatively, use the submersible magnetic stirrer apparatus, B(g).

(3) *Incubation with pancreatic α-amylase/AMG*.—Incubate the reaction solutions at 150 revolutions/min (orbital motion) or at the relevant rate to ensure suspension in a reciprocal shaker at 37°C for 16 h ± 10 min (e.g., 5:00 pm to 9:00 am). Alternatively, stir the contents of the Fisherbrand bottles, B(b), on a 2 mag Mixdrive 15 stirring device (with 7 × 30 mm stirrer bar) submerged in a water bath at 37°C for 16 h ± 10 min (Figure 2011.25A).

(4) *Adjustment of pH to approximately 8.2 (pH 7.9–8.4), inactivation of α-amylase and AMG*.—After 16 h, remove sample bottles from the shaking water bath and immediately add 3.0 mL

of 0.75 M Trizma base solution to terminate the starch digestion reactions. (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.) Slightly loosen the caps of the sample bottles and place the bottles in a water bath (nonshaking) at 95–100°C, and incubate for 20 min with occasional shaking (by hand). Using a thermometer, ensure that the final temperature of the bottle contents is >90°C (checking of just one bottle is adequate).

(5) *Cool*.—Remove all sample bottles from the hot water bath (use appropriate gloves) and cool to approximately 60°C.

(6) *Protease treatment*.—Add 0.1 mL protease solution, C(f), using a positive displacement dispenser (solution is viscous). Incubate at 60°C for 30 min.

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

(8) *Addition of internal standard*.—To each sample, add 1 mL of 100 mg/mL internal standard solution, C(q).

(9) Proceed to G(a) for determination of IDF when IDF and SDF values are desired. Proceed to O(a) when TDF is desired without fractionation to IDF and SDF.

G. Determination of IDF

(a) *Filtration setup*.—Tare crucible containing Celite to nearest 0.1 mg. Wet and redistribute the bed of Celite in the crucible, using



Figure 2011.25A. Typical arrangement for sample mixing using a 2 mag Mixdrive 15 submersible magnetic stirrer in a custom made water bath.

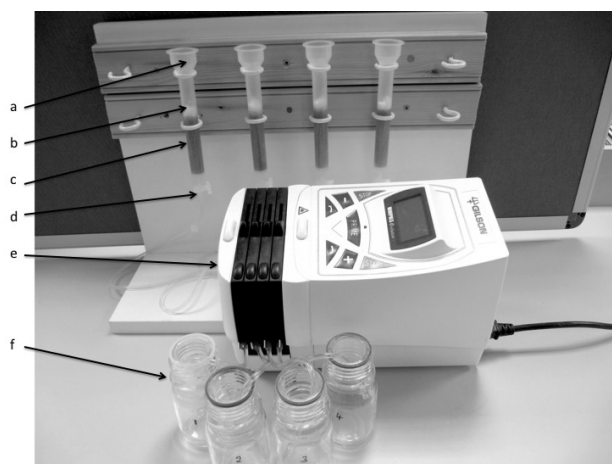


Figure 2011.25B. Column and pump arrangement for deionization of sample extracts: (a) Bio-Rad Econo-Pac® disposable chromatography column (Cat. No. 732-1010); (b) cotton wool plug; (c) a mixture of approximately 4 g Ambersep 200(H⁺) resin and approximately 4 g Amberlite FPA53 (OH⁻) resin; (d) stopcock, one-way tap; (e) Gilson Minipuls® Evolution pump; (f) Duran bottle collection containers.

15 mL of 78% (v/v) EtOH (or IMS), **C(b)**, from wash bottle. Apply suction to crucible to draw Celite onto fritted glass as an even mat. Discard this filtrate.

(b) *Filtration.*—Using vacuum, filter enzyme digest, **F(b)**(9), through crucible. With the aid of a wash bottle use 21.9 mL or less of deionized water (warmed to 60°C) to quantitatively transfer remaining particles to crucible and rinse residue. Retain combined filtrate and transfer water and set aside for determination of water SDF, **H(a)**.

(c) *Wash.*—Using vacuum, wash residue two times each with 15 mL portions of 78% ethanol, 95% ethanol, and acetone. (*Note:* Delay in washing IDF residues with 78% ethanol, 95% ethanol, and acetone may cause inflated IDF values.) Discard these washings.

(d) Dry crucibles containing residue overnight in 105°C oven.

(e) Cool crucible in desiccator for approximately 1 h. Weigh crucible containing IDF residue and Celite to nearest 0.1 mg. To obtain residue mass, subtract tare weight (i.e., weight of dried crucible and Celite).

(f) *Protein and ash determination.*—Residue from one crucible of the duplicates is analyzed for protein, and the second residue of the duplicates is analyzed for ash. Perform protein analysis on residue using Kjeldahl or combustion methods. (Caution should be exercised when using a combustion analyzer for protein in the residue. Celite volatilized from the sample can clog the transfer lines of the unit.) Use 6.25 factor for all cases to calculate mg 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.

(g) Proceed to determination of water soluble dietary fiber, **H(a)**.

H. Determination of Water Soluble Dietary Fiber

(a) *Precipitation of water soluble SDFP.*—To each sample filtrate, add any remaining water from the IDF transfer and rinse, **G(b)**, or additional water if necessary to bring the total volume to exactly 70 mL, then add 279 mL (measured at room

temperature) of 95% (v/v) EtOH or IMS, **C(a)**, preheated to 60°C and mix thoroughly. Allow the SDFP precipitate to form at room temperature for 60 min.

(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, **C(b)**, from wash bottle. Apply suction to crucible to draw Celite onto fritted glass as an even mat. Discard this filtrate.

(c) *Filtration.*—Using vacuum, filter precipitated SDFP, **H(a)**, from supernatant through crucible. Using a wash bottle with 78% (v/v) EtOH or IMS, **C(b)**, quantitatively transfer all remaining particles to crucible. Retain filtrate and washings, (c) and (d), respectively, for determination of SDFS, **I(d)**.

(d) *Wash.*—Using a vacuum, wash residue successively with two 15 mL portions of the following: 78% (v/v) EtOH or IMS; 95% (v/v) EtOH or IMS; acetone. Combine with filtrate of previous step.

(e) Dry crucibles containing residue overnight in 105°C oven.

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

(g) *Protein and ash determination.*—Residue from one crucible of the duplicates is analyzed for protein, and the second residue of the duplicates is analyzed for ash. Perform protein analysis on residue using Kjeldahl or combustion methods. (Caution should be exercised when using a combustion analyzer for protein in the residue. Celite volatilized from the sample can clog the transfer lines of the unit.) Use 6.25 factor for all cases to calculate mg 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 **I(d)**.

I. Determination of SDFS

Chromatography of maltodextrins, maltose, glucose, sorbitol, and glycerol on a Sugar-Pak column is shown in Figure 2011.25E and of water:alcohol soluble dietary fiber (WASDF or SDFS), glucose, and sorbitol in Figure 2011.25F. *Note:* Proper deionization is an essential part of obtaining quality chromatographic data on SDFS. For manual deionization, be sure to obtain familiarity regarding the appearance of salt peaks in the SDFS chromatograms which may interfere with dietary fiber determination. In evaluating the column containing approximately 8 g of mixed bed resin, dissolve 10 mg sodium chloride into 9 mL deionized water and

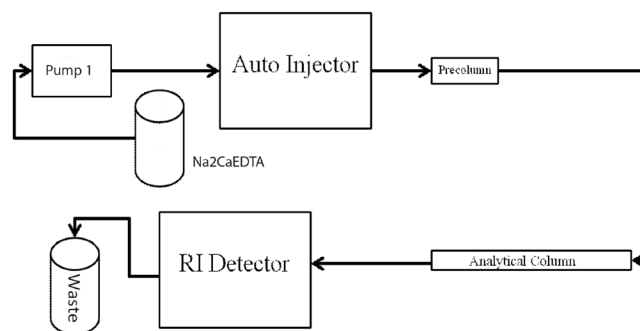


Figure 2011.25C. Apparatus assembly of liquid chromatography system for Sugar-Pak analytical column—manual deionization.

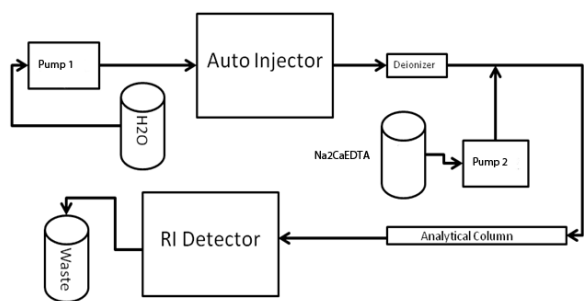


Figure 2011.25D. Apparatus assembly of liquid chromatography system for Sugar-Pak analytical column—simultaneous online deionization and quantitation.

add 1 mL of 100 mg/mL LC internal standard, C(q), and proceed to I(e) at “transfer 2 mL to the top of the column of...”. The liquid chromatogram of this solution should show no peaks in the time range corresponding to carbohydrates of DP ≥ 3 . For simultaneous online deionization and quantitation by LC, see Figure 2011.25F regarding the change in chromatography which occurs as the ion exchange capacity of the cartridges is exceeded.

(a) *LC system setup.*—Set up the LC system choosing between the setups shown schematically in Figures 2011.25C and D depending upon whether manual or online deionization is used.

Sugar-Pak column operating conditions are: (1) *Temperature.*—90°C.

(2) *Manual deionization-mobile phase.*—Distilled water plus Na₂CaEDTA (50 mg/L); flow rate, 0.5 mL/min.

(3) *Simultaneous online deionization and quantitation.*—Mobile phase for pump 1, distilled water; flow rate, 0.45 mL/min. Mobile phase for pump 2, Na₂CaEDTA (500 mg/L); flow rate,

0.05 mL/min.

(4) System must clearly separate maltose from higher maltodextrins (Figure 2011.25E).

(5) *Run time.*—30 min to ensure that all materials from the injection are cleared from the column prior to the next injection.

(6) *RI detector at 50°C.*—*Note:* For samples containing significant amounts of short-chain polyfructose, it is recommended that chromatography of the SDFS be carried out on 2-size exclusion LC columns (TSK-Gel 30 cm \times 7.8 mm) connected in series (Sigma Aldrich Part No. 808020) using conditions described in AOAC *Official Method*SM 2009.01.

(b) *Calibrate the area of chromatogram to be measured for SDFS.*—Use a 100 μ L LC syringe, B(aa), to fill the 50 μ L injection loop with retention time standard, C(o). Determine demarcation point between DP 2 and 3 oligosaccharides (disaccharides sucrose and maltose versus higher oligosaccharides). See Figures 2011.25E and F.

(c) *Determine response factor (Rf) for D-glucose.*—Since D-glucose provides an LC RI response equivalent to the Rf of the nondigestible polysaccharides that make up SDFS, the LC is calibrated using D-glucose, and the Rf is used for determining the mass of SDFS. Use a 100 μ L LC syringe, B(aa), to fill the 50 μ L injection loop for each internal standard/D-glucose solution, C(r).

(1) *Internal standard method.*—Obtain the values for the peak areas of D-glucose and internal standard from the chromatograms. The reciprocal of the slope obtained by comparing the ratio of peak area of D-glucose/peak area of internal standard (*y*-axis) to the ratio of the mass of D-glucose/mass of internal standard (*x*-axis) is the “response factor.” Determine the average Rf (typically 0.91 relative to D-sorbitol internal standard).

$$\text{Response factor (Rf)} = \frac{(\text{PA-IS})/(\text{PA-Glu}) \times (\text{Wt-Glu}/\text{Wt-IS})$$

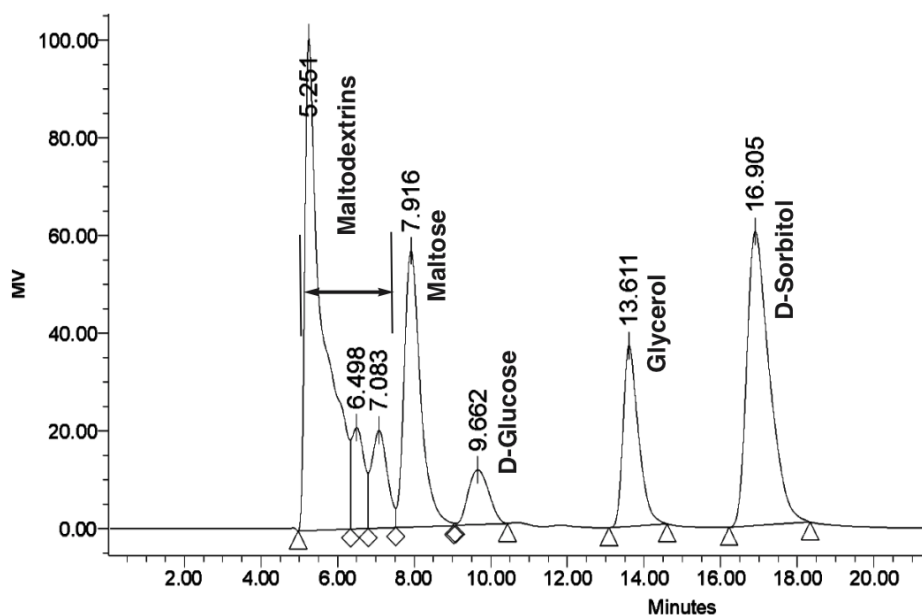


Figure 2011.25E. Chromatography of a mixture of maltodextrins, maltose, D-glucose, D-sorbitol, and glycerol on a Waters Sugar-Pak column. Right arrow shows demarcation between DP 2 (maltose) and DP 3 (higher maltodextrins).

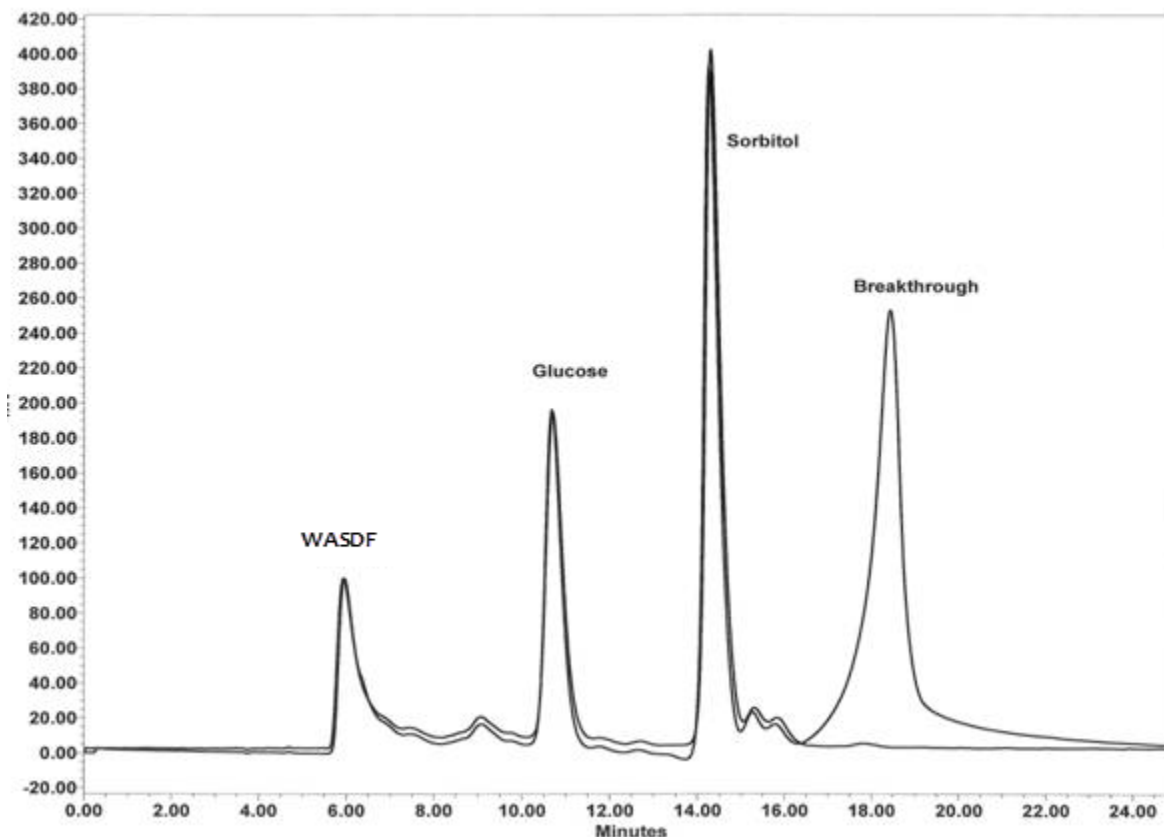


Figure 2011.25F. Chromatogram of typical SDFS in sample on a Sugar-Pak column analyzed before and after the capacity of the deionizing cartridges has been expended. Note the absence of the peak at 18.4 min when the deionizing cartridges are functioning properly, and appearance of the ion peak at 18.4 min indicating the need for replacement of cartridges.

where PA-IS = peak area internal standard (D-sorbitol); PA-Glu = peak area D-glucose; Wt-Glu = mass of D-glucose (mg) contained in 1 mL of standard (5, 10, or 20 mg); Wt-IS = mass of internal standard (mg) contained in 1 mL of standard (10 mg D-sorbitol).

(2) *External standard method.*—Obtain the values for the peak areas of D-glucose from the three chromatograms. Determine the average Rf:

$$Rf = (Wt-Glu)/(PA-Glu)$$

where Wt-Glu = mass (mg) of D-glucose contained in 1 mL of standard (5, 10, or 20 mg); PA-Glu = peak area D-glucose.

(d) *Filtrate recovery, deionization, and LC analysis.*—(Set aside the filtrate from one of the sample duplicates, **H(c)**, to use in case of spills or take it through the procedure if duplicate SDFS data is desired.) Transfer filtrate(s), **H(c)**, of the other sample duplicate to a 1 L evaporator flask and concentrate with a rotary evaporator to dryness at 50°C. Dissolve the residue in 10 mL deionized water, and transfer to a sealable container and store at 4°C if planning to store overnight before deionization. (In subsequent work, half of the filtrate was concentrated to dryness and redissolved in 5 mL water. This, as expected gave the same results, but halved the time for rotary evaporation.) For manual deionization, proceed to **I(e)**. For simultaneous online LC deionization and quantitation, proceed to **I(f)**.

(e) *Deionization of sample.*—In the interlaboratory study, manual deionization was performed using separate columns of Ambersep 200 (H⁺) resin and Amberlite FPA53 (OH⁻) resins (10 g of each) in Alltech Extract-Clean reservoirs (15 mL) connected with an Alltech Syringe Adapter (Cat. No. 210705). However, with subsequent lots of columns and adaptors, a perfect connection was not obtained and solutions were lost, so it was decided to use mixed bed resin [Ambersep 200 (H⁺) and Amberlite FPA53 (OH⁻)] (4 g of each) in a Bio-Rad Econo-Pac disposable chromatographic column (Cat. No. 732-1010) and to reduce sample size by the same percentage (40%; i.e., from 5 to 2 mL). Since the ratio of sample and resins remained the same, identical results were obtained. Weigh approximately 4 g Ambersep 200 (H⁺) and approximately 4 g Amberlite FPA53 (OH⁻) resins into a 100 mL beaker and slurry with a minimum volume of distilled water. Apply this slurry to a Bio-Rad Econo-Pac column and use distilled water from a wash bottle to transfer all resin to the column. Add a small cotton wool plug to the top of the resin and wash the column contents with approximately 20 mL deionized water. Connect the column to a Gilson Minipuls® Evolution pump (<http://www.gilson.com/en/Pipette/Products/53.229>, or similar). Centrifuge or filter the sample solution and transfer 2 mL to the top of the column of mixed bed resin and allow the column to elute at approximately 1 mL/min. Collect the eluate in a 250 mL round-bottom, rotary evaporator flask. When the liquid level has dropped to the level of the cotton wool plug, rinse the sample in with 2 mL

distilled water and allow this to percolate into the resin collecting the eluate. Then add approximately 20 mL distilled water to the top of the column and allow this to elute into the 250 mL round-bottom flask at approximately 1 mL/min. Collect the eluate until the liquid level reaches the top of the resin in the column. Evaporate the solution just to dryness at 50°C. Dissolve the sample to bring it up to 2 mL of volume with distilled water by swirling the flask by hand for approximately 2 min. Transfer the sample into a polypropylene storage tube using a Pasteur pipet.

(f) *Preparation of samples for LC analyses.*—Transfer the solutions from either (d) or (e) to a 10 mL disposable syringe, **B(z)**, and filter through a 0.45 µm filter, **B(w)**.

(g) *HPLC of sample solutions.*—Use a 100 µL LC glass syringe, **B(aa)**, to fill the 50 µL injection loop on the LC system, **B(u)(2)**, with filtered sample extracts. For manually deionized samples from **I(e)** after filtration step, **I(f)**, apply solutions via the injection loop, **B(u)(2)**, directly onto the Waters Sugar-Pak column, **B(u)(3)**, using the arrangement shown in Figure 2011.25C. For samples to be deionized online from **I(d)** after filtration step, **I(f)**, apply solutions to the injection loop and the online desalting guard columns, **B(t)**, using the arrangement shown in Figure 2011.25D.

(h) *Determine peak area of SDFS (PA-SDFS) and internal standard if used (PA-IS) in chromatograms of sample extracts.*—Record area of all peaks of DP greater than the DP 2/DP 3 demarcation point as PA-SDFS. Record the peak area of internal standard as PA-IS.

J. Calculations for IDF

(a) *Blank (B, mg) determination.*

$$B = [(BR1 + BR2)]/2 - P_B - A_B$$

where BR1 and BR2 = blank residue mass (mg) for duplicate IDF blank determinations, respectively, and P_B and A_B = mass (mg) of protein and ash, respectively, determined on first and second blank residues.

(b) *IDF.*

$$\text{IDF, mg/100 g} = [(R1 + R2)/2 - P - A - B]/(M1 + M2)/2 \times 100$$

$$\text{IDF, \%} = \text{IDF (mg/100 g)/1000}$$

where R1 = IDF residue mass 1 from M1 in mg; R2 = IDF residue mass 2 from M2 in mg; M1 = test portion mass 1 in g; M2 = test portion mass 2 in g; A = ash mass (mg) from R1; P = protein mass (mg) from R2; B = IDF blank.

K. Calculations for SDFP

(a) *Blank (B, mg) determination.*

$$B = [(BR1 + BR2)]/2 - P_B - A_B$$

where BR1 and BR2 = blank residue mass (mg) for duplicate SDFP blank determinations, respectively, and P_B and A_B = mass (mg) of protein and ash, respectively, determined on first and second blank residues.

(b) *SDFP.*

$$\text{SDFP, mg/100 g} =$$

$$[(R1 + R2)/2 - P - A - B]/(M1 + M2)/2 \times 100$$

$$\text{SDFP, \%} = \text{SDFP (mg/100 g)/1000}$$

where R1 = SDFP residue mass 1 from M1 in mg; R2 = SDFP residue mass 2 from M2 in mg; M1 = test portion mass 1 in g; M2 = test portion mass 2 in g; A = ash mass (mg) from R1; P = protein mass (mg) from R2; B = SDFP blank.

L. Calculations for SDFS

(a) *Internal standard method.*—[Note: Sorbitol is present in some foods and food products, such as plums (prunes), pears, apples, and polydextrose and may also be added to foods. If sorbitol is present, use of the internal standard will result in artificially low SDFS quantity. In these cases, the external standard method of calculation should be used. Addition of the internal standard is still recommended to use as a retention time reference.]

$$\text{SDFS, mg/100 g} = R_f \times (\text{Wt-IS, mg}) \times (\text{PA-SDFS})/(\text{PA-IS}) \times 10 \times 100/M$$

$$\text{SDFS, \%} = \text{SDFS (mg/100 g)/1000}$$

where R_f = response factor calculated in **I(c)**; Wt-Glu = mass (mg) of D-glucose contained in 1 mL of standard (5, 10, or 20 mg); PA-Glu = peak area D-glucose; Wt-IS is mass of internal standard in 1 mL of sample (i.e., 10 mg per mL; final sample volume, 10 mL); PA-SDFS is the peak area of the water:alcohol soluble dietary fiber; PA-IS is the peak area of the internal standard; 10 is factor to convert from 1 to 10 mL (final sample volume); 100 is factor to convert to 100 g of sample; M is the test portion mass (g) M1 or M2 of the sample whose filtrate was concentrated and analyzed by LC.

(b) *External standard method.*

$$\text{SDFS, mg/100 g} = R_f \times (\text{PA-SDFS}) \times 10 \times 100/M$$

$$\text{SDFS, \%} = \text{SDFS (mg/100 g)/1000}$$

where R_f = (Wt-Glu)/(PA-Glu); Wt-Glu = mass (mg) of D-glucose contained in 1 mL of standard (5, 10, or 20 mg); PA-Glu = peak area for D-glucose (5, 10, or 20 mg); PA-SDFS is the peak area of the water:alcohol soluble dietary fiber; 10 is factor to convert from 1 to 10 mL (final sample volume); 100 is factor to convert to 100 g of sample; M is the test portion mass (g) M1 or M2 of the sample whose filtrate was concentrated and analyzed by LC.

M. Calculations for SDF

$$\text{SDF, mg/100 g} = \text{SDFP (mg/100 g)} + \text{SDFS (mg/100 g)}$$

$$\text{SDF, \%} = \text{SDF (mg/100 g)/1000}$$

N. Calculation for TDF

$$\text{TDF, \%} = \text{IDF (\%)} + \text{SDF (\%)}$$

O. Determination of TDF Without Fractionation of IDF and SDF

(a) *Precipitation of water soluble SDFP in the presence of IDF.*—To each sample enzyme digest, from step **F(b)(9)**, add water to bring the total volume to exactly 71 mL. Then add 279 mL (measured at room temperature) of 95% (v/v) EtOH or IMS, **C(a)**, preheated to 60°C and mix thoroughly. Allow the SDFP precipitate to form in the beaker with the IDF at room temperature for 60 min.

(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, **C(b)**, from wash bottle. Apply suction to crucible to draw Celite onto fritted glass as an even mat. Discard this filtrate.

(c) *Filtration.*—Using vacuum, filter precipitated SDFP:IDF, **(a)**, from supernatant through crucible. Using a wash bottle with 78% (v/v) EtOH or IMS, **C(b)**, quantitatively transfer all remaining particles to crucible. Retain filtrate and washings, **(c)** and **(d)**, for determination of low-molecular-weight SDF, **I(d)**.

(d) *Wash and retain filtrate.*—Using a vacuum, wash residue successively with two 15 mL portions of the following: 78% (v/v) EtOH or IMS, followed by two 15 mL portions of 95% (v/v) EtOH or IMS, followed by two 15 mL portions of acetone. Combine with filtrate from step immediately preceding.

(e) Dry crucibles containing residue overnight in 105°C oven.

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

(g) *Protein and ash determination.*—Residue from one crucible of the duplicates is analyzed for protein, and the second residue of the duplicates is analyzed for ash. Perform protein analysis on residue using Kjeldahl or combustion methods. (Caution should be exercised when using a combustion analyzer for protein in the residue. Celite volatilized from the sample can clog the transfer

lines of the unit.) Use 6.25 factor for all cases to calculate mg 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 **I(d)** for determination of SDFS.

P. Calculations for SDFP:IDF

(a) *Blank (B, mg) determination.*

$$B = [(BR1 + BR2)]/2 - P_B - A_B$$

where BR1 and BR2 = blank residue mass (mg) for duplicate SDFP:IDF blank determinations, respectively, and P_B and A_B = mass (mg) of protein and ash, respectively, determined on first and second blank residues.

(b) *SDFP:IDF.*

$$\text{SDFP:IDF, mg/100 g} = \frac{[(R1 + R2)/2 - P - A - B]}{(M1 + M2)/2} \times 100$$

$$\text{SDFP:IDF, \%} = \text{SDFP:IDF (mg/100 g)/1000}$$

where R1 = SDFP:IDF residue mass 1 from M1 in mg; R2 = SDFP:IDF residue mass 2 from M2 in mg; M1 = test portion mass 1 in g; M2 = test portion mass 2 in g; A = ash mass (mg) from R1; P = protein mass (mg) from R2; B = blank.

Q. Calculation for TDF

$$\text{TDF, \%} = \text{SDFP:IDF (\%)} \text{ (from P)} + \text{SDFS (\%)} \text{ (from L)}$$

Reference: *J. AOAC Int.* (future issue)