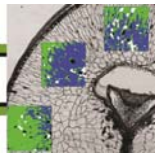

e-Xtra*

Measurement of Total Dietary Fiber Using AOAC Method 2009.01 (AACC International Approved Method 32-45.01): Evaluation and Updates

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ABSTRACT

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The Codex Committee on Methods of Analysis and Sampling recently recommended 14 methods for measurement of dietary fiber, eight of these being type I methods. Of these type I methods, AACC International Approved Method 32-45.01 (AOAC method 2009.01) is the only procedure that measures all of the dietary fiber components as defined by Codex Alimentarius. Other methods such as the Prosky method (AACCI Approved Method 32-05.01) give similar analytical data for the high-molecular-weight dietary fiber contents of food and vegetable products low in resistant starch. In the current work, AACCI Approved Method 32-45.01 has been modified to allow accurate measurement of samples high in particular fructooligosaccharides: for example, fructotriose, which, in the HPLC system used, chromatographs at the same point as disaccharides, meaning that it is currently not included in the measurement. Incubation of the resistant oligosaccharides fraction with sucrase/β-galactosidase removes disaccharides that interfere with the quantitation of this fraction. The dietary fiber value for resistant starch type 4 (RS_4), varies significantly with different analytical methods, with much lower values being obtained with AACCI Approved Method 32-45.01 than with 32-05.01. This difference results from the greater susceptibility of RS_4 to hydrolysis by pancreatic α-amylase than by bacterial α-amylase, and also a greater susceptibility to hydrolysis at lower temperatures. On hydrolysis of samples high in starch in the assay format of AACCI Approved Method 32-45.01 (AOAC method 2009.01), resistant maltodextrins are produced. The major component is a heptasaccharide that is highly resistant to hydrolysis by most of the starch-degrading enzymes studied. However, it is hydrolyzed by the maltase/amyloglucosidase/isomaltase enzyme complex present in the brush border lining of the small intestine. As a consequence, AOAC methods 2009.01 and 2011.25 (AACCI Approved Methods 32-45.01 and 32-50.01, respectively) must be updated to include an additional incubation with amyloglucosidase to remove these oligosaccharides.

Based on the recommendation for endorsement of the Codex Committee on Nutrition and Foods for Special Dietary Uses in November 2008 (Codex Alimentarius 2008), a definition for dietary fiber (DF) was adopted in June 2009 by the Codex Alimentarius Commission (Codex Alimentarius 2010). The definition lists three categories of carbohydrates that are not hydrolyzed by the endogenous enzymes in the small intestine of humans. However, the definition left the decision concerning the inclusion or otherwise of oligosaccharides with degrees of polymerization (DPs) in the range of 3–9 to the discretion of national authorities and left the "physiological effect(s) of benefit to health" as undefined (Howlett et al 2010) (Box 1).

Codex Alimentarius defines four distinct classes of methods, from defining methods to tentative methods, each with its own range of applicability (Box 2) (Codex Alimentarius 1997). At its 32nd session in Budapest in March 2011, the Codex Committee on Methods of Analysis and Sampling (2012) approved 14 methods for the measurement of DF: eight as type I methods, five as type II, and one as type III. These are listed in Table I, along with the associated AACCI Approved Method number, the AOAC International method number, and a summary of what the method measures. Included in this table is AOAC method 2011.25 (AACCI Approved Method 32-50.01) (McCleary et al 2012), which has not yet been considered by Codex Alimentarius because the AOAC/AACCI interlaboratory study has just been completed. This method separately measures insoluble DF and higher-molecular-weight soluble DF (HMWSDF), as well as lower-molecular-weight soluble DF (LMWSDF). Of the eight methods recommended for measurement of "total dietary fiber," only one of

BOX 1

Codex Alimentarius Definition of Dietary Fiber

Dietary fiber consists of carbohydrate polymers with ten or more monomeric units, which are not hydrolyzed by the endogenous enzymes in the small intestine of humans and belong to the following categories: edible carbohydrate polymers naturally occurring in the food as consumed; carbohydrate polymers which have been obtained from food raw material by physical, enzymatic or chemical means and which have been shown to have a physiological effect of benefit to health as demonstrated by generally accepted scientific evidence to competent authorities, and; synthetic carbohydrate polymers which have been shown to have a physiological effect of benefit to health as demonstrated by generally accepted scientific evidence to competent authorities.

a. When derived from a plant origin, dietary fiber may include fractions of lignin and/or other compounds when associated with polysaccharides in the plant cell walls and if these compounds are quantified by the AOAC gravimetric analytical method for dietary fiber analysis: fractions of lignin and the other compounds (proteic fractions, phenolic compounds, waxes, saponins, phytates, cutin, phytosterols, etc.) intimately "associated" with plant polysaccharides in the AOAC 991.43 method.

b. Decision on whether to include carbohydrates of 3 to 9 monomeric units should be left up to national authorities.

Codex Alimentarius (2010)

* The *e*-Xtra logo stands for "electronic extra" and indicates that images of equipment used and a supplemental video are available online.

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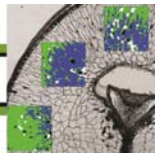


BOX 2

Four Types of Methods

Codex Alimentarius methods are defined as follows:

Type I (Defining Methods)—"A method which determines a value that can only be arrived at in terms of the method per se and serves by definition as the only method for establishing the accepted value for the item measured."

Type II (Reference Methods)—"A Type II method is the one designated Reference Method where Type I methods do not apply. It should be selected from Type III methods (as defined below). It should be recommended for use in cases of dispute and for calibration purposes."

Type III (Alternative Approved Methods)—"A Type III method is one which meets the criteria required by the Codex Committee on Methods of Analysis and Sampling for methods that may be used for control, inspection or regulatory purposes."

Type IV (Tentative Method)—"A Type IV method is one which has been used traditionally or else has been recently introduced but for which the criteria required for acceptance by the Codex Committee on Methods of Analysis and Sampling have not yet been determined."

Codex Alimentarius (1997)

these, AOAC method 2009.01 (AACC International Approved Method 32-45.01), measures the total content of DF, including resistant starch (RS) and nondigestible oligosaccharides (NDO) (also called LMWSDF), as defined by Codex.

In the measurement of the DF components, ethanol is added to a concentration of 76% to precipitate the HMWSDF from lower-molecular-weight DF fractions (LMWSDF or NDO). It is erroneously believed that precipitation in 76% aqueous ethanol separates oligosaccharides of DP of 9 (soluble) from those of DP of 10 and greater (insoluble). No single-step precipitation can fractionate soluble oligomeric carbohydrates that precisely. To move away from this perception, we (McCleary et al 2012) have renamed the DF fractions as insoluble DF (IDF), DF soluble in water but insoluble in 76% aqueous ethanol (SDFP), and DF soluble in water and also soluble in 76% aqueous ethanol (SDFS). This nomenclature will be used throughout this article (Box 3).

BOX 3

Definition of Dietary Fiber (DF) Terminology

IDF	Insoluble DF.
SDFP	DF soluble in water but insoluble in 76% aqueous ethanol.
SDFS	DF soluble in water and soluble in 76% aqueous ethanol.
HMWDF	High-molecular-weight DF = IDF + SDFP.
TDF	Total DF = HMWDF (IDF + SDFP) + SDFS.
LMWSDF	Low-molecular-weight soluble DF.
NDO	Nondigestible oligosaccharides.
SDF	Soluble DF (SDF) = LMWSDF = NDO.

In the evaluation of AOAC method 2009.01 (AACCI Approved Method 32-45.01), several issues have arisen concerning the analysis of some samples, including problems in the measurement of highly degraded inulin, "underestimation" of RS type 4 (RS_4; phosphate cross-linked starch), and measurement of maltodextrins derived from native starch or starch in bread and other samples that are resistant to hydrolysis in the assay procedure. These issues are addressed in this article, and the DF values for a range of samples determined by using several methods are compared.

MATERIALS AND METHODS

Materials

Test kits for the measurement of total DF (K-TDFR; AOAC methods 985.29 and 991.43), integrated total DF (K-INTDF; AOAC methods 2009.01 and 2011.25), RS (K-RSTAR; AOAC method 2002.02), total starch (K-TSTA; AOAC method 996.01), α-amylase (K-CERA; AOAC method 2002.01), Beta-Glucazyme test tablets (T-BGZ200), pancreatic α-amylase (E-PANAA), *Rhizopus* amyloglucosidase (AMG) (E-AMGPU), *Aspergillus niger* AMG (E-AMGDF), thermostable bacterial α-amylase (E-BLAAM), pullulanase (E-PULBL), isoamylase (E-ISAMY), α-glucosidase (*Bacillus stearothermophilus*; E-TSAGL), and β-galactosidase (*A. niger*; E-BGLAN) were obtained from Megazyme (Bray, Ireland). Barley β-glucan (medium viscosity; catalog number P-BGBM) and wheat arabinoxylan (catalog number P-WAXM) were from Megazyme. Crystalline papain (16–40 U/mg) (catalog number P3125-100MG) was from Sigma Chemical Company (St. Louis, MO, U.S.A.). Partially degraded chicory inulin (Raftilose P-95) was a kind gift from CRDS-Tienen (Central Department Research, Development and Services, Südzucker Group, Tienen, Belgium). Regular maize starch was from Penford Australasia (Lane Cove, NSW, Australia). Hylon VII (ref. 98GH8401), Novelose 330 (ref. AH17529), and Novelose 240 (ref. 96LF10063) were from National Starch and Chemical Company (Bridgewater, NJ, U.S.A.). Native potato starch was from Avebe (Foxhol, The Netherlands). ActiStar (enzyme-modified tapioca/cassava starch; U.S. patent 6,043,229) was

TABLE I

Codex/AOAC/AACCI Methods for the Analysis of Dietary Fiber (DF)

AOAC Method	AACCI Method	Codex Type[a]	What Is Measured[b]
985.29	32-05.01	I	Total HMWDF (IDF + HMWSDF)
991.42	32-20.01	I	IDF in foods
993.19	…	I	HMWSDF in foods
991.43	32-07.01	I	IDF and HMWSDF separately
994.13	32-25.01	I	Total HMWDF; provides sugar composition and Klason lignin
2001.03	32-41.01	I	HMWDF and LMWSDF in foods devoid of resistant starch
993.21	…	I	Total HMWDF in samples with >10% fiber and <2% starch
2009.01	32-45.01	I	HMWDF and LMWSDF in all foods
2011.25	32-50.01	*	IDF, HMWSDF, and LMWSDF in all foods
995.16	32-23.01	II	(1→3)(1→4)-β-Glucan in cereals, feeds, and foods
997.08	32-31.01	II	Fructans and FOS
999.03	32-32.01	III	Fructans and FOS (underestimates highly depolymerized FOS)
2000.11	32-28.01	II	Polydextrose
2001.02	32-33.01	II	Trans galacto-oligosaccharides
2002.02	32-40.01	II	Resistant starch (RS_2 and RS_3)

[a] Asterisk (*) indicates that no decision has yet been made by Codex concerning this method. Method types are defined in Box 2.

[b] HMWDF = higher-molecular-weight DF; IDF = insoluble DF; HMWSDF = higher-molecular-weight soluble DF; LMWSDF = lower-molecular-weight soluble DF; and FOS = fructooligosaccharides.

from Cerestar (Vilvoorde, Belgium). FiberRite and Fibersym were from MGP Ingredients (Atchison, KS, U.S.A.).

Dry food and bean samples were obtained and prepared as follows: dried red lentils (Rainbow Wholefoods, Kylemore Industrial Estate, Dublin, Ireland), dried haricot and pinto bean (The Health Store, Nottingham, U.K.), and corn flakes (Kellogg Co., Tesco Supermarket, Greystones, Ireland) were milled to pass a 0.5 mm screen with a Fritsch Pulverisette 14 mill (Fritsch, Idar Oberstein, Germany). Alternative mills that grind materials to pass a 0.5–0.7 mm screen can be used. Moisture contents were determined for all samples by AOAC method 925.10 (AOAC 2007).

Wet bean, vegetable, and fruit samples were obtained and prepared as follows. Potato (local fruit and vegetable store) was cooked and cooled before lyophilization. Green banana (local fruit and vegetable store) was minced with a standard mechanical meat mincer (Porkert, Prague, Czech Republic) with a 4.5 mm screen and lyophilized. Canned red kidney beans (Chivers Ireland Ltd., Coolock, Dublin, Ireland) were recovered on a food strainer and washed with water. They were then minced (4.5 mm screen) and lyophilized. All lyophilized material was milled to pass a 1.0 mm screen with a Fritsch Pulverizette 14 mill before analysis.

General Methods

TLC was performed on Merck DC-Alufolien Kieselgel 60 (0.2 mm pre-prepared plates). Aliquots (10 µL) of oligosaccharides (10 mg/mL) were applied to the plates, and the plates were developed once with 7:1:2 *n*-propanol–ethanol–water. Spots were detected by spraying with 5% sulfuric acid in ethanol and heating at 110°C in a small oven. HPLC was performed using an HPLC with an oven to maintain a column temperature of 90°C and a 50 µL injection loop. The column was a Waters Sugar Pak 6.5 by 300 mm (part number WAT085188) or equivalent. Operating conditions were temperature, 90°C; mobile phase, distilled water plus EDTA (50 mg/L); and flow rate, 0.5 mL/min.

Measurement of DF

Four methods were employed in this study: AACCI Approved Method 32-05.01/AOAC method 985.29 (Prosky et al 1985), AACCI Approved Method 32-07.01/AOAC method 991.43 (Lee et al 1992), AACCI Approved Method 32-45.01/AOAC method 2009.01 (McCleary 2007; McCleary et al 2010), and AACCI Approved Method 32-50.01/AOAC method 2011.25 (McCleary et al 2012). A broad overview of these methods is given in Figure 1,

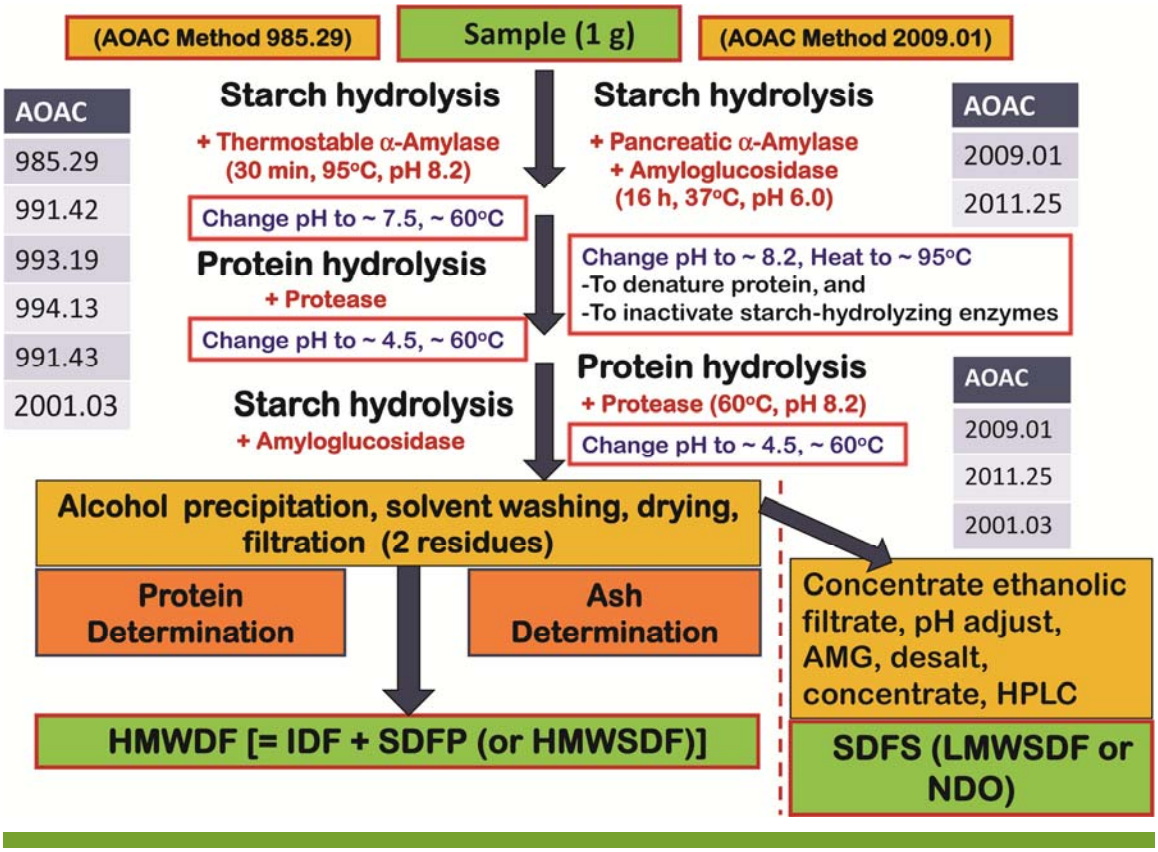
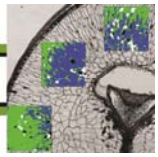


Fig. 1. Pictorial representation of the steps involved in the AOAC dietary fiber (DF) methods (Codex type I methods). Equivalent AACCI Approved Methods are listed in Table I. On the left side of the figure are methods based on extensions of AOAC method 985.29. On the right hand side are the two methods based on use of pancreatic α-amylase under physiological conditions (AOAC methods 2009.01 and 2011.25). In the middle right of the figure, the methods that incorporate measurement of DF soluble in water and also soluble in 76% aqueous ethanol (SDFS) are listed. AMG = amyloglucosidase; IDF = insoluble DF; SDFS = DF soluble in water and soluble in 76% aqueous ethanol; and SDFP = DF soluble in water but insoluble in 76% aqueous ethanol.



and enzymes and buffers employed are detailed in Table II. In all methods, only highly purified enzymes (essentially devoid of contaminating activities) were employed. Enzyme purity is essential when analyzing such a diverse material as DF, including RS and NDO (SDFS). Some commercially available AMG enzyme preparations routinely used in DF determination are highly contaminated with β-glucanase (cellulase). This conatmination leads to depolymerization and, thus, can lead to underestimation of DF in β-glucan-containing grains, food products, and ingredients. This contamination is clearly demonstrated by viscometry with medium-viscosity barley β-glucan as substrate (Fig. 2). Alternatively, this contamination can be shown with Beta-Glucazyme test tablets, which specifically measure enzymes active on mixed-linkage β-glucans. A second essential requirement of enzymes used in DF determination is that they meet the activity requirements stated in the official methods. In at least one case, the concentration of protease supplied in a commercial kit for DF measurement is less than 10% of that required in the AOAC official method. Low levels of protease activity can lead to limited hydrolysis of protein, resulting in high protein contents of residues and increased errors in determinations. Basically, it is important that enzymes have the required activity and purity so that nonfiber components are removed and that there is no hydrolysis and loss of the DF components.

RS was analyzed according to AOAC method 2002.02. To determine the RS in the residue obtained in AOAC methods 2009.01 and 985.29 (AACCI Approved Methods 32-45.01 and 32-05.01), the methods were altered slightly; on addition of the four volumes of industrial methylated spirits (IMS), the precipitates were recovered by centrifugation (10,000 rpm, 10 min), and the pellets washed twice with 100 mL of 76% IMS and then analyzed for RS following the same procedure as in AOAC method 2002.02 but scaled up 10-fold. Moisture content of samples was determined following AOAC method 925.10 (AOAC 2007).

Modified Method for SDFS Determination

SDFS obtained from high-starch-containing samples such as cereal grains, bread, and pasta analyzed following AACCI Approved Methods 32-45.01 and 32-50.01 (AOAC methods 2009.01 and 2011.25) contains branched maltodextrins that are resistant to hydrolysis by the levels of pancreatic α-amylase and AMG used in the assay but have been shown to be hydrolyzed by the α-glucosidase complex in the small intestine of pigs (and, most likely, humans). These oligosaccharides were removed as follows. First, half of the aqueous ethanol solution obtained on filtration of the hydrolyzed sample in ethanol was concentrated to dryness by rotary evaporation. The residue was dissolved in 5 mL of 150mM HCL (to give a pH of approximately 4.5) and transferred to a polypropylene tube. An aliquot (0.1 mL, 330 U) of AMG was added, and the tube was sealed and incubated at 60°C for 1 h and then at approximately 100°C for 5 min. An aliquot (2 mL) of this solution was added to a column of mixed-bed resin (4 g of Amberlite FPA53 [OH^-] resin plus 4 g of Ambersep 200 [H^+]) in Bio-Rad Econo-Pac disposable chromatography columns (catalog

TABLE II

Summary of the Enzymes and Buffers Used in Various AOAC (Codex Type I) Dietary Fiber (DF) Methods

AOAC Method	What Is Measured [a]	Enzymes	Specific Features
985.29	Total HMWDF (IDF + SDFP)	Bacterial α-amylase, protease, and AMG	Phosphate buffer, 1 g sample
991.42	IDF in foods	Same as 985.29	Phosphate buffer, 1 g sample
993.19	SDFP in foods	Same as 985.29	Phosphate buffer, 1 g sample
991.43	IDF and SDFP separately	Same as 985.29	MES/Tris buffer, pH 8.2, 1 g sample
994.13	HMWDF; provides sugar composition and Klason lignin	Same as 985.29, but no protease	Acetate buffer, 250–500 mg sample
2001.03	HMWDF and SDFS in foods devoid of resistant starch	Same as 985.29	Phosphate buffer, 1 g sample
993.21	HMWDF in samples with >10% fiber and <2% starch	No enzymes	No buffer, 0.1 g sample
2009.01	HMWDF and SDFS in all foods	Pancreatic α-amylase, protease, and AMG	Maleate buffer, pH 6, 1 g sample
2011.25	IDF, SDFP, and SDFS in all foods	Pancreatic α-amylase, protease, and AMG	Maleate buffer, pH 6, 1 g sample

[a] HMWDF = higher-molecular-weight DF; IDF = insoluble DF; SDFP = DF soluble in water but insoluble in 76% aqueous ethanol; SDFS = DF soluble in water and also soluble in 76% aqueous ethanol; and AMG = amyloglucosidase. Total dietary fiber = HMWDF + SDFS. HMWDF = IDF + SDFP.

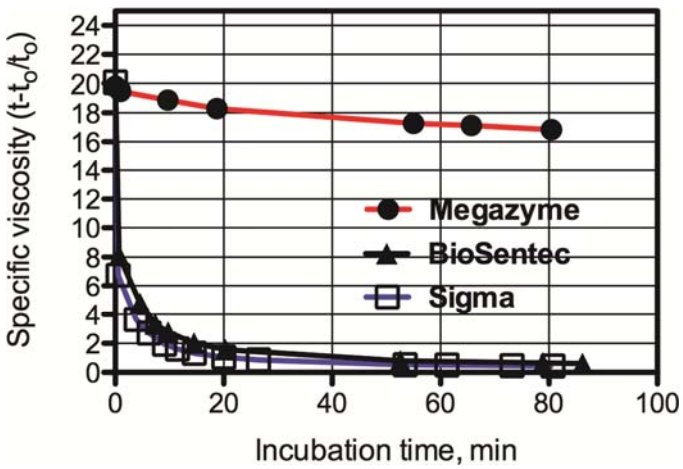
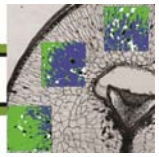


Fig. 2. Determination of β-glucanase contamination in commercially available amyloglucosidase (AMG) preparations as used in dietary fiber analysis according to AOAC method 985.29 (AACCI Approved Method 32-05.01). In each case, the amount of AMG used in the assay is the amount recommended by the suppliers for use in AOAC method 985.29. The AMG was added to a solution of barley β-glucan (10 mL, 10 mg/mL, 25 cSt) in 100mM sodium acetate buffer (pH 4.5) in an Ostwald C-type viscometer suspended in a water bath at 40°C. The time of flow was recorded at various time intervals and the specific viscosity calculated and plotted. Megazyme is lot 51002; Sigma is Sigma Chemical Company, lot A9913-10ML 119K8719; and BioSentec is lot 32C-11181.



number 732-1010) with an Alltech one-way stopcock (catalog number 211524), as previously described (McCleary et al 2010). The solution was allowed to elute at 1 mL/min, and then 2 mL of distilled water was added to the column to wash in the sample; the column was then washed with 20 mL of distilled water at 1 mL/min. The eluate was concentrated to dryness and redissolved in 2 mL of water, filtered through a 0.45 µm filter, and analyzed by HPLC.

Measurement of Nonresistant Starch in RS_4

Incubation conditions were the same as in AOAC method 2009.01 (AACCI Approved Method 32-45.01). Sample (1.00 g) was added to a 250 mL Fisherbrand soda-glass wide-mouth bottle followed by a magnetic stirrer bar (7 × 30 mm) and 40 mL of buffer containing the enzymes being evaluated. Buffer/enzyme combinations employed were 1) pancreatic α-amylase (50 U/mL); 2) pancreatic α-amylase (50 U/mL) plus AMG (3.4 U/mL), both in 50mM sodium maleate buffer (pH 6.0) plus 2mM calcium chloride and 0.02% sodium azide; and 3) bacterial α-amylase as per AOAC method 985.29 (50 µL of 10,000 U/mL, i.e., 12.5 U/mL in the incubation buffer) in MES/Tris buffer (50mM of each, pH 8.2). The reaction solutions were incubated at 37°C with stirring by a 2mag Mixdrive 15 submersible magnetic stirrer (2mag Magnetic Motion, Munich, Germany). Samples (10 mL) were removed from the stirring incubation solutions and immediately filtered through Whatman number 1 (9 cm) filter circles. Aliquots (1 mL) were diluted 26-fold in 0.1M sodium acetate buffer (pH 4.5) and mixed thoroughly. An aliquot (0.1 mL, in duplicate) was transferred to the bottom of a glass test tube, 0.1 mL of AMG (30 U/mL) was added, and the tubes were incubated at 40°C for 10 min to hydrolyze maltodextrins to glucose. Glucose was determined by the addition of 3.0 mL of glucose oxidase/peroxidase (GOPOD) reagent (from the glucose test kit) with stirring and then incubation at 40°C for 20 min. The absorbance at 510 nm was measured for all samples along with glucose standards (100 µg in quadruplicate) and blanks. Nonresistant starch was determined as grams per 100 g of sample on an "as-is" basis and on a "dry weight" basis allowing for the moisture content of the sample (Fig. 3) and presented here as degree of hydrolysis (%). RS was calculated by difference, allowing for the fact that the starch samples were approximately 98% pure.

The effect of incubation temperature on the extent of hydrolysis of starch samples by bacterial α-amylase was studied as follows. Aliquots (40 mL) of MES/Tris buffer in capped 250 mL Fisherbrand bottles were preincubated for 20 min at 40, 50, 60, 70, 80, or 100°C with stirring by a 2mag Mixdrive 15 submersible magnetic stirrer. Starch sample (1.00 g) was added and allowed to disperse with stirring over 2 min. Thermostable bacterial α-amy-

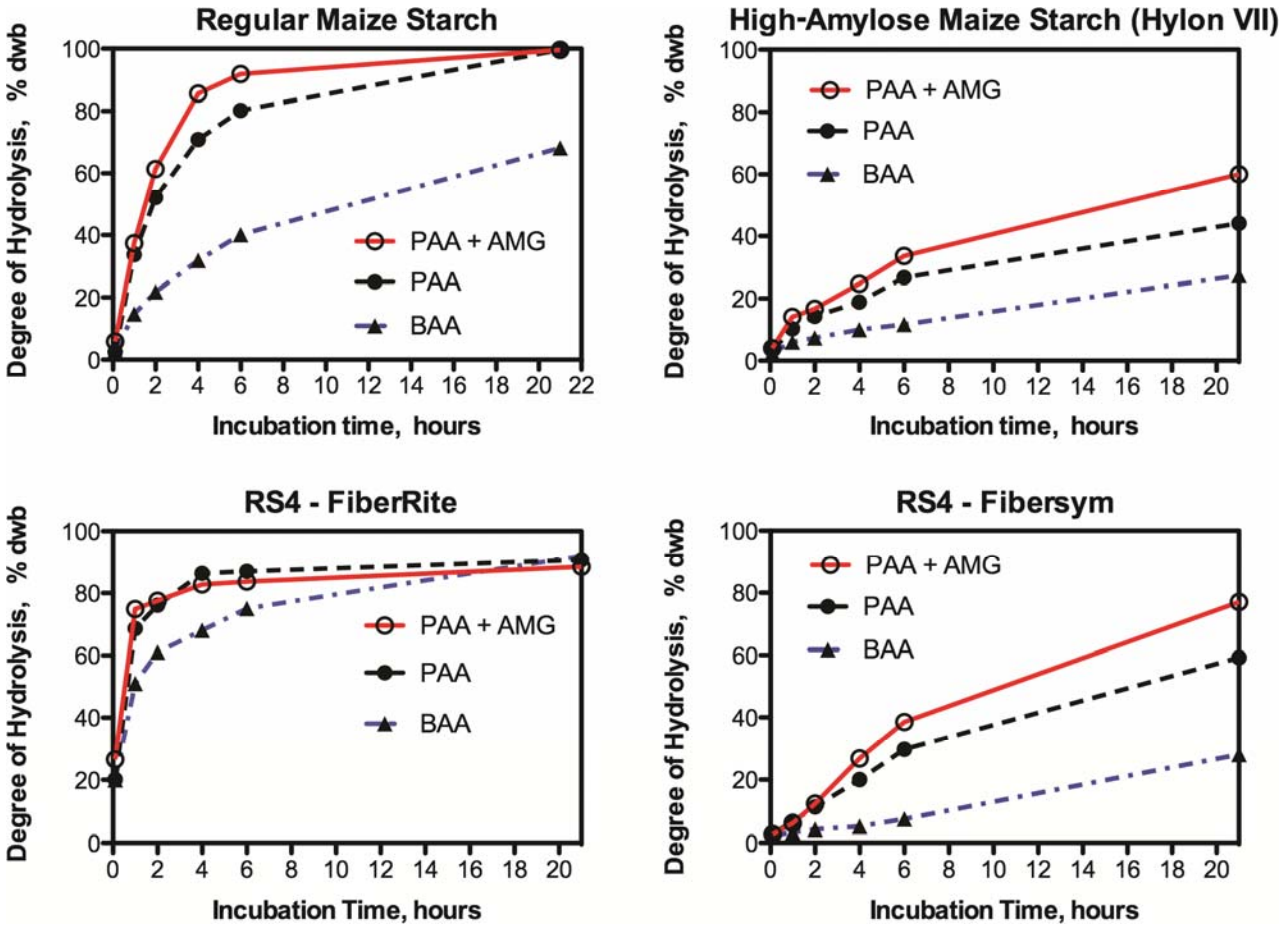
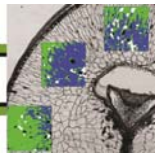


Fig. 3. Hydrolysis of regular maize starch, high-amylose maize starch, and two commercial resistant starch (RS_4) samples (FiberRite and Fibersym) by pancreatic (PAA) and bacterial α-amylases (BAA). Experimental conditions are described in Materials and Methods. AMG = amyloglucosidase.



lase (50 µL, 10,000 U/mL) as used in AOAC method 985.29 was added, and stirring continued at 170 rpm for 1 h. An aliquot (10 mL) of the reaction mixture was immediately filtered through Whatman number 1 filter paper. Aliquots (1 mL) of the filtrate were diluted 26-fold in 0.1M sodium acetate buffer (pH 4.5), samples (0.1 mL in duplicate) were incubated with AMG, and glucose was determined using GOPOD reagent, as described previously. Soluble starch was determined from the glucose value, and the degree of hydrolysis (solubilization of the insoluble starch granules) was calculated and plotted (Fig. 4).

In another experiment (Table III), incubations were performed following AOAC method 2009.01, but one set of samples was preincubated in buffer in a boiling water bath for 30 min before cooling to 37°C and addition of enzymes. Sample (1.00 g) was added to a Fisherbrand bottle followed by 20 mL of 50mM sodium maleate buffer (pH 6) plus $CaCl_2$ and NaN_3. The bottle and contents were incubated in a boiling water bath for 30 min, with occasional swirling of the contents. The bottles were removed from the bath and cooled to 37°C. Then, 20 mL of maleate buffer containing pancreatic α-amylase (100 U/mL) plus AMG (6.8 U/mL) was added to give the same enzyme concentration as in AOAC method 2009.01. The containers were sealed and incubated with stirring for 16 h at 37°C. Nonresistant starch was determined as described earlier and RS calculated by difference.

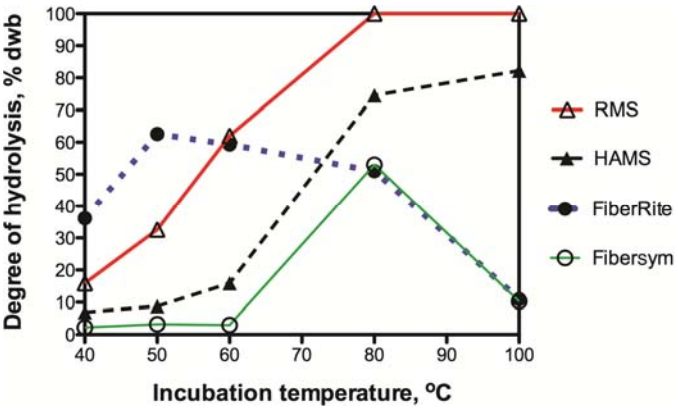


Fig. 4. Effect of incubation temperature on the extent of hydrolysis of regular maize starch (RMS), high-amylose maize starch (HAMS), and two commercial resistant starch (RS_4) samples (FiberRite and Fibersym) by bacterial α-amylase. Experimental conditions are described in Materials and Methods.

Preparation of Resistant Heptasaccharide, Tetrasaccharide, and Octa-/Nonasaccharides from Hydrolyzed Maize Starch

Regular maize starch (40 g) was suspended in 1.6 L of pancreatic α-amylase/AMG-buffered solution as used in AOAC method 2009.01, and the mixture was incubated with stirring in a water bath at 37°C for 16 h. The preparation was heated in a microwave oven to approximately 90°C to inactivate enzymes, cooled to room temperature, and desalted by passage through a column (5 × 30 cm) of a mixed-bed resin of equal quantities of Amberlite FPA (OH^-) (Megazyme catalog number G-AMBOH) and Ambersep 200 (H^+) (Megazyme catalog number G-AMBH). The eluate was concentrated to approximately 120 mg/mL of carbohydrate and applied in 20 mL aliquots to a column of Bio-Gel P-2 (<400 mesh) (5 × 95 cm) (Bio-Rad Laboratories, Hercules, CA, U.S.A.) at 60°C in degassed, deionized water. The fractions rich in tetrasaccharide, heptasaccharide, and octa-/nonasaccharides were separately collected, concentrated to near dryness, and lyophilized.

Enzymic Hydrolysis of Maltodextrins

A series of experiments were performed.

Experiment 1. An aliquot (0.1 mL) of maltodextrin (50 µg) was added to 0.1 mL of 100mM sodium acetate buffer (pH 4.5). Reaction was initiated by adding 0.1 mL of *A. niger* AMG (20 U) and incubating at 40°C for 10 min. Reaction was terminated and released glucose determined by adding 3.0 mL of GOPOD reagent and incubating at 40°C for 20 min. In a parallel experiment, maltodextrin (0.1 mL, 50 µg) was added to 0.1 mL of 100mM sodium phosphate buffer (pH 6.0) followed by 0.1 mL of *B. stearothermophilus* α-glucosidase (16 U). Incubations were performed the same way with glucose determination with GOPOD reagent (Table IV).

Experiment 2. An aliquot (0.1 mL) of maltodextrin (50 µg; maltoheptaose or resistant maltodextrin, DP 7) was added to 0.1 mL of 100mM sodium acetate buffer (pH 4.5), and 0.1 mL of AMG (0.02–20 U) was added. The mixture was incubated at 40°C for 10 min and reaction terminated and glucose determined with GOPOD reagent (Table V).

Experiment 3. An aliquot (0.1 mL) of the resistant heptasaccharide (50 µg) recovered from regular maize starch or whole grain bread was added to 0.1 mL of 100mM sodium acetate buffer (pH

TABLE III

Nonresistant and Resistant Starch (RS) Values Determined for Regular Maize Starch, High-Amylose Maize Starch (Hylon VII), and Two RS_4 Starch Samples (Fibersym and FiberRite)[a]

Sample	Preincubation (≈100°C, 30 min)	Nonresistant Starch (g/100 g, as is)	Moisture Content (%)	Nonresistant Starch (g/100 g, dwb)	Resistant Starch (g/100 g, dwb)
Regular maize starch	–	92.9	5.2	97.7	2.3
Regular maize starch	+	92.8	5.2	97.6	2.4
High-amylose maize starch	–	44.7	10.0	49.2	50.8
High-amylose maize starch	+	69.0	10.0	75.9	24.1
Fibersym RS	–	62.5	10.5	69.0	31.0
Fibersym RS	+	82.5	10.5	94.5	5.5
FiberRite RS	–	78.6	9.6	86.1	13.9
FiberRite RS	+	84.7	9.6	92.8	7.2

[a] Samples were analyzed according to AOAC method 2002.02, with measurement of nonresistant starch (as glucose) rather than measurement of RS. One set of samples was preheated to 95°C for 30 min before analyzing according to AOAC method 2009.01. RS was determined by difference; "as is" indicates analyzed directly; and "dwb" indicates values adjusted based on moisture content of the samples.

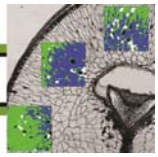


TABLE IV

Hydrolysis of Maltodextrins and Resistant Maltodextrin Degree of Polymerization 7 (RMD7) by *Aspergillus niger* Amyloglucosidase (AMG) (Highly Purified) and *Bacillus stearothermophilus* α-Glucosidase (Ultrapure)[a]

	Hydrolysis (%)	
Oligosaccharide	*A. niger* AMG (20 U/assay)	*B. stearothermophilus* α-Glucosidase (16 U/assay)
RMD7 from regular maize starch	100	2.8
RMD7 from whole grain bread	90	1.7
Maltose	100	100
Maltopentaose	100	100
Maltohexaose	100	100
Maltoheptaose	100	100
Isomaltose	5	22

[a] An aliquot (0.1 mL) of AMG or α-glucosidase was added to a solution of maltodextrin (0.1 mL, 50 µg) plus 0.1 mL of 100mM buffer (pH 4.5, 5.0, or 6.0) and the mixture was incubated at 40°C for 10 min. The reaction was terminated and released glucose determined by addition of GOPOD reagent (see Materials and Methods).

TABLE V

Hydrolysis of Maltoheptaose and Resistant Maltodextrin Degree of Polymerization 7 (RMD7) by *Aspergillus niger* Amyloglucosidase (AMG)[a]

	Hydrolysis of Oligosaccharides (%)		
AMG (U/incubation)	Maltoheptaose	RMD7 from Regular Maize Starch	RMD7 from Whole Grain Bread
20.0	100	100	90
2.0	100	27	20.0
0.2	78	2.1	1
0.02	32	1.0	0.3

[a] An aliquot (0.1 mL) of AMG (0.02–20.0 U) was added to a solution of maltodextrin (0.1 mL, 50 µg) plus 0.1 mL of 100mM buffer (pH 4.5), and the mixture was incubated at 40°C for 10 min. The reaction was terminated and released glucose determined by addition of GOPOD reagent (see Materials and Methods).

5.0) or 100mM sodium phosphate buffer (pH 6.0). To this was added 0.1 mL of a range of enzymes, including *A. niger* AMG (20 U), *Rhizopus* AMG (20 U), *B. stearothermophilus* α-glucosidase (16 U), pancreatic α-amylase (150 U) plus *B. stearothermophilus* α-glucosidase (16 U), *A. niger* α-amylase (100 U) plus *B. stearothermophilus* α-glucosidase (16 U), pullulanase (30 U) plus *B. stearothermophilus* α-glucosidase (16 U), or isoamylase (50 U) plus *B. stearothermophilus* α-glucosidase (16 U). The pH of incubation for each of these enzyme mixtures is shown in Table VI. The mixtures were incubated at 40°C for 10 min, GOPOD reagent added, and free glucose determined. Degree of hydrolysis (%) was calculated as glucose released as a percentage of total carbohydrate (phenol-sulfuric acid method) (Dubois et al 1956).

Extraction, Solubilization, and Assay of Maltodextrin-Degrading Enzymes from the Brush Border Lining of the Small Intestine of Pig

Recovery of Pig Intestinal Tissue and Extraction and Solubilization of Enzymes. Small intestines from several pigs were provided by Liam Quelly, Irish Dog Foods Ltd. The intestines were collected fresh from an abattoir and shipped on ice. The mucosal enzymes were extracted and assayed on the day of collection of the tissue. Three 30 cm sections of upper intestine were prepared by cutting the tissue with a surgical knife. The sections were turned inside out and the tissue laid flat on a polypropylene lid over crushed ice. Mucosal cells were removed by scraping the tissue with a microscope slide. The mucosal suspension (10 mL) was transferred to a polypropylene tube with a Pasteur pipette, and 1.2 mL of 0.5M sodium maleate buffer (pH 6.0), 0.65 mL of freshly prepared cysteine hydrochloride solution (10 mg/mL), and 0.15 mL of crystalline papain suspension (4 mg, 16–40 U/mg) were added. The suspension was incubated at 37°C for 1 h. During this period, most of the insoluble cellular material was solubilized. The solution was centrifuged (10,000 × *g*, 15 min) to remove a trace of insoluble material before use in enzyme assays. A supplemental video of this process is available online, along with a PDF showing images of equipment used.

Assay of Enzyme Activities. An aliquot (0.1 mL) of a range of oligosaccharides (10 mg/mL) in 100mM sodium maleate buffer (pH 6.0) was transferred to the bottom of a glass test tube and preincubated at 40°C for 3 min. Papain-treated mucosal solution (0.1 mL) was added with stirring on a vortex mixer. Tubes were incubated for 0, 3, 6, 9, and 12 min at 40°C, and the reaction was terminated by placing the tube into a boiling water bath for 2 min. Glucose was determined by adding 3 mL of GOPOD reagent (Megazyme K-GLUC) and incubating at 40°C for 20 min. Absorbances were measured at 510 nm.

Purification of Fructotriose from Raftilose P-95

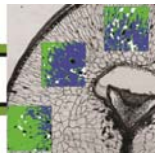
Fructosyl-β(2-1)-fructosyl-β(2-1)fructose (F3) was purified from Raftilose P-95 by chromatography on Bio-Gel P-2 at 60°C. A second oligosaccharide (kestose) was present as a minor contaminant (approximately 5%, w/w).

Hydrolysis of Sucrose, Maltose, and Lactose in the Presence of F3

An aliquot (0.1 mL) of a mixture of α-glucosidase (43 U) and β-galactosidase (50 U) (Megazyme catalog number E-SUCRBG diluted sixfold in distilled water) was added to an aliquot (1.0 mL) of 5mM sodium acetate buffer solution (pH 5.0) containing sucrose (5 mg/mL), maltose (5 mg/mL), lactose (5 mg/mL), F3 (5 mg/mL), or various mixtures of these sugars in a glass tube and incubated at 40°C for 1 h. The reaction was terminated by heating the tube in a boiling water bath for 2 min. The solution was transferred to a microcentrifuge tube and centrifuged at 12,000 rpm for 5 min, and the supernatant solution was analyzed by HPLC with a Waters Sugar Pak column.

Characterization of the Structure of the Resistant Maltoheptaoside Derived on Hydrolysis of Regular Maize Starch in AOAC Method 2009.01

GC/MS Linkage Analysis. For glycosyl linkage analysis, the sample was permethylated, depolymerized, reduced, and acetylated, and the resulting partially methylated alditol acetates analyzed by GC-MS, as described by York et al (1985). Initially, dry sample was suspended in approximately 300 µL of dimethyl sulfoxide and placed on a magnetic stirrer for five days. The sample was then permethylated by the method of Ciucanu and Kerek (1984) (treatment with sodium hydroxide and methyl iodide in dry dimethyl sulfoxide). The sample was subjected to the NaOH base



for 10 min; then, methyl iodide was added and left for 40 min. The base was then added for 10 min and, finally, more methyl iodide was added for 40 min. This addition of more methyl iodide and NaOH base was to insure complete methylation of the oligomer. Following sample workup, the permethylated material was hydrolyzed with 2M trifluoroacetic acid (2 h in a sealed tube at 121°C), reduced with $NaBD_4$, and acetylated with acetic anhydride/trifluoroacetic acid. The resulting partially methylated alditol acetates were analyzed on a Hewlett Packard 5975C GC interfaced to a 7890A mass selective detector (electron impact ionization mode); separation was performed on a 30 m Supelco 2330 bonded phase fused silica capillary column.

Electrospray Ionization MS Analysis. Electrospray ionization (ESI)-MS analysis was performed with an LTQ Orbitrap XL mass spectrometer (Thermo-Fisher Waltham, MA, U.S.A.) equipped with a nanospray ion source. The sample (50 µg) was dissolved in 10mM ammonium hydroxide solution in 50% methanol and infused directly into the instrument at a constant flow rate of 0.5 µL/min. The capillary temperature was set at 210°C, and MS analysis was performed in the negative ion mode. A full Fourier transform mass spectrometry spectrum was collected at 30,000 resolution. For MS/MS analysis, the collision energy was set at 30%.

NMR Analysis. The sample was deuterium-exchanged by lyophilization from D_2O, dissolved in D_2O, and transferred to an NMR tube with a 5 mm outer diameter. Proton-proton and proton-carbon correlated spectra were acquired on a Varian Inova-800 MHz spectrometer equipped with a 5 mm cryoprobe. All spectra were acquired at 25°C. Chemical shifts were referenced to internal acetone ($\delta[^1H]$ = 2.20 ppm, $\delta[^{13}C]$ = 31.07 ppm). All experiments (1D proton, 2D gradient correlation spectroscopy [gCOSY], total correlation spectroscopy [TOCSY], gradient heteronuclear single quantum coherence [gHSQC], heteronuclear multiple bond correlation [HMBC], and nuclear Overhauser enhancement spectroscopy [NOESY]) were acquired with standard Varian pulse sequences.

RESULTS AND DISCUSSION

Codex Alimentarius Methods

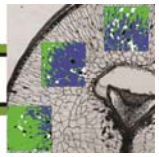
The most basic requirement for the accurate and reliable measurement of DF with any official method is that the defined enzymes are used and that they match up to the activity and purity requirements stated in the AOAC official methods. This requirement is clearly demonstrated in Figure 2, in which the level of contaminating β-glucanase in AMG preparations currently supplied for DF determination is shown. Clearly, two of the preparations contain significant levels of β-glucanase, resulting in a rapid decrease in viscosity of barley β-glucan. Significant depolymerization will lead to an underestimation of this polysaccharide, particularly in food matrices in which it occurs as less than 10% of the sample weight. Protease hydrolysis is included in AOAC DF methods to reduce protein in residues so that, when weight corrections are made, errors caused by this component are minimized. Detailed work by Prosky and coworkers (1985) established an acceptable level (approximately 350 U/mL of protease solution used), as defined in the AOAC methods. The level of activity in a protease supplied by BioSentec (lot 32 Ra-11181, 27 U/mL) was less than 10% of that required in official methodology.

Some details of the enzymes, buffers, and conditions used in the AOAC/Codex type 1 methods are summarized in Table II and shown graphically in Figure 1. AOAC methods 985.29, 991.42, 993.19, 991.43, 994.13, and 2001.03 (AACCI Approved Methods 32-45.01, 32-20.01, 32-07.01, 32-25.01, and 32-41.01) all employ thermostable α-amylase, protease, and AMG. The conditions of incubation with the thermostable α-amylase (approximately 95°C) result in hydrolysis and, thus, loss of a percentage of RS in samples containing this component. AOAC methods 2009.01 and 2011.25 (AACCI Approved Methods 32-45.01 and 32-50.01) involve incubation with pancreatic α-amylase under physiological conditions of temperature and pH and, thus, result in a more accurate estimation of RS, as shown by data given in Table VII. In this

TABLE VI
Hydrolysis of Resistant Maltodextrin Degree of Polymerization 7 (RMD7) by Various Starch-Degrading Enzymes[a]

Enzyme	Hydrolysis of Oligosaccharide (%)		Hydrolysis of Starch	
	RMD7 from Regular Maize Starch	RMD7 from Whole Grain Bread	Glycosidic Bonds Cleaved (µmol/10 min)	Theoretical Excess Overactivity Required (-fold)
Amyloglucosidase (*Aspergillus niger*) (20 U)[b]	97	90	200	648
Amyloglucosidase (*Rhizopus*) (20 U)[b]	102	90	200	648
α-Glucosidase (*Bacillus stearothermophilus*) (16 U)[c]	3	1.5	160	520
Pancreatic α-amylase (150 U)	3.0	1.5	1,500	4,860
Plus α-glucosidase (*B. stearothermophilus*) (16 U)[c]				520
A. niger α-amylase (100 U)	1.9	1.5	1,000	3,240
Plus α-glucosidase (*B. stearothermophilus*) (16 U)[c]				520
Isoamylase (50 U)	2.6	3	500	1,620
Plus α-glucosidase (*B. stearothermophilus*) (16 U)[b]				520
Pullulanase (30 U)	2.4	1.6	300	972
Plus α-glucosidase (*B. stearothermophilus*) (16 U)[c]				520
Invertase (300 U)[b]	0	0	3,000 (sucrose)	…

[a] An aliquot (0.1 mL) of enzyme was added to a solution of maltodextrin (0.1 mL, 50 µg) plus 0.1 mL of 100mM buffer (pH 5 or 6) and the mixture was incubated at 40°C for 10 min. The reaction was terminated and released glucose determined by addition of GOPOD reagent (see Materials and Methods).
[b] pH 5.
[c] pH 6.



table, RS values for a range of samples analyzed with the AOAC RS method (2002.02) and DF method 2009.01 are compared. Using AOAC method 2009.01, RS values in the DF residues were determined following the same procedure as in AOAC method 2002.02 to recover and dissolve the RS, but scaled up 10-fold. Also compared are HMWDF values obtained with AOAC DF methods 2009.01 and 991.43. Clearly, there is a good agreement between RS values for all samples except potato starch, Actistar, and green banana. The lower values obtained with AOAC method 2009.01 compared with 2002.02 are because, in 2009.01, samples are heated in a boiling water bath to denature protein (essential for protease digestion). In this step, some of the RS is solubilized, and recovery requires effective precipitation in the presence of 76% ethanol. Of course, it is also essential that the starch-degrading enzymes are inactivated before the RS solubilizes. For AMG, this inactivation is achieved by the pH change to 8.2, at which pH the enzyme is effectively inactive, followed by complete inactivation at temperatures above 65°C. Pancreatic α-amylase would have activity at pH 8.2, but this enzyme is inactivated at approximately 50°C, a temperature well below that at which RS is solubilized (60–70°C). In AOAC method 2002.02, the RS is not solubilized at all. Native potato starch and Actistar cannot be used as a source of RS in food products that are heated because they will dissolve and no longer function as RS. HMWDF values obtained following AOAC methods 991.43 and 2009.01 are also compared in Table VII. With native potato starch and Actistar, the HMWDF values obtained with AOAC method 991.43 are close to zero. The HMWDF values for green banana, Hylon VII, dry milled pinto bean, and haricot bean are also dramatically reduced because of solubilization and hydrolysis of RS. A more detailed description of what is measured by the various AOAC/AACCI methods is shown in Figure 5 and Table I, and the details listed in Table VIII assist the analyst in choosing which method to use, based on the sample being analyzed and what DF components are to be measured. AOAC method 2009.01 measures everything shown in Figure 5, and the problems of double counting experienced when summing values obtained with AOAC methods 985.29 and 2002.02 (RS), for example, are avoided. In analyzing phosphate cross-linked starch (RS_4) with AOAC method 2009.01, lower values are obtained than with AOAC method 985.29. This difference is not because of an underestimation of RS by 2009.01 but, rather, because of an overestimation by 985.29, as will be discussed later in this article.

Analytical data for a range of samples obtained using four DF methods are given in Tables IX and X. HMWDF values measured directly (AOAC method 985.29) or measured as SDFP and insoluble DF summed (AOAC method 991.43) are shown in Table IX. As expected, the values are quite similar even though, in AOAC method 991.43, there are many more analytical steps. A comparison of values obtained with AOAC methods 2009.01 and 2011.25 is given in Table X. HMWDF contents determined by the two methods are very similar. Also, values obtained with AOAC methods 985.29 and 2009.01 (Table X) are in good agreement for samples not containing RS. Consequently, if AOAC method 2009.01 is adopted as a reference method, as has been done in Canada (www.hc-sc.gc.ca/fn-an/consult/fibre-fibres/consul-fibre-fibres-eng.php), HMWDF values obtained following AOAC methods 985.29 or 991.43 remain valid. Traditionally, DF values obtained

TABLE VII

Comparison of Resistant Starch (RS) and HMW Dietary Fiber (DF) Values Obtained for a Number of Samples Using AOAC Methods 2002.02, 2009.01, and 991.43

	AOAC Methods			
	RS (%, w/w)[a]		HMWDF (%, w/w)	
Sample	2002.02	2009.01	2009.01	991.43
Native potato starch	64.9	56.8	64.6	0.9
Actistar	58.0	48.8	47.3	0.5
Green banana	51.0	38.0	37.6	7.5
Hylon VII	50.0	48.6	49.3	25.6
Novelose 240	48.4	44.2	44.3	47.1
Novelose 330	38.8	38.7	39.9	35.0
Pinto bean (dry milled)	39.4	35.6	54.9	17.3
Haricot bean (dry milled)	36.9	31.2	51.9	23.3
Red kidney bean	5.0	5.3	21.8	20.4
Red lentils	7.6	6.1	14.8	11.3
Cooked/cooled potato	4.0	3.2	9.6	7.1
Kellogg's Corn Flakes	2.2	2.4	3.7	2.9
Regular maize starch	0.5	0.8	0.7	0.1

[a] RS contents in the residues obtained in AOAC method 2009.01 were measured using an adaption of AOAC method 2002.02 (see Materials and Methods).

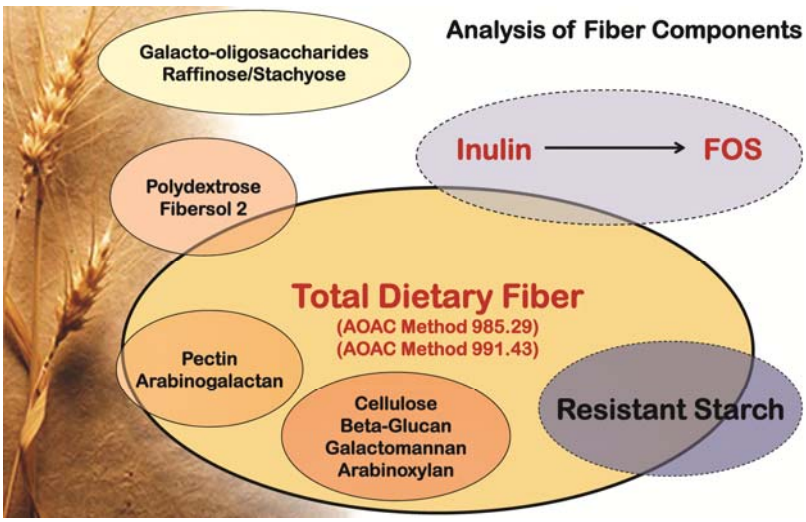
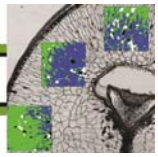


Fig. 5. Schematic representation of dietary fiber components measured and not measured by AOAC official methods 985.29 and 991.43 (AACCI Approved Methods 32-05.01 and 32-07.01). Also depicted are the problems of partial measurement of resistant starch, polydextrose, and resistant maltodextrins by current AOAC total dietary fiber methods. Most of the dietary fiber that is soluble in water and also soluble in 76% aqueous ethanol (galactooligosaccharides, fructooligosaccharides [FOS], and so on) is not measured. AOAC methods 2009.01 and 2011.25 (AACCI Approved Methods 32-45.01 and 32-50.01, respectively) measure all components shown, with no double counting.



following AOAC method 985.29 have been referred to as "total dietary fiber," but clearly this terminology is no longer relevant because the DF soluble in aqueous ethanol (SDFS) has not been included. It is recommended that the fiber component measured by AOAC methods 985.29 and 991.43 be referred to as high-molecular-weight dietary fiber (HMWDF).

The number of analytical steps in AOAC 2009.01 is similar to that in AOAC 985.29 if SDFS is not measured (Fig. 1). The only significant difference is that, in 2009.01, the α-amylase/AMG incubation step is performed for 16 h (overnight), under physiological conditions of pH and temperature. AOAC method 2009.01 offers the advantages that it is applicable to all samples and incu-

TABLE VIII

Measurement of Dietary Fiber (DF): Which AOAC/AACCI Method to Use[a]

Sample, Fiber Component of Interest	AOAC (AACCI) Method
Plant and food material not containing RS	
HMWDF (IDF plus SDFP)	985.29 (32-05.01) or 991.43 (32-07.01) (with IDF and SDFP together) or 994.13 (32-25.01) (if sugar composition is required)
IDF and SDFP (separately)	991.43 (32-07.01)
IDF	991.43 (32-07.01) (discarding SDFP component) or 991.42 (32-20.01)
SDFP	991.43 (discarding IDF component) or 993.19
HMWDF and SDFP (separately)	2001.03
IDF, SDFP, and SDFS (separately)	991.43 (32-07.01) (for IDF and SDFP) plus SDFS format from 2001.03 (32-41.01) or 2009.01 (32-45.01)
Plant and food materials with or without RS	
HMWDF (including RS) and SDFS (i.e., all dietary fiber)	2009.01 (32-45.01)
IDF (containing RS), SDFP and SDFS	2011.25 (32-50.01)

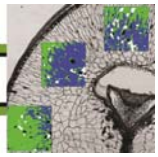
[a] RS = resistant starch; IDF = insoluble DF; SDFP = DF soluble in water but insoluble in 76% aqueous ethanol; and SDFS = DF soluble in water and also soluble in 76% aqueous ethanol.

TABLE IX

Comparison of Dietary Fiber (DF) Values Obtained Using AOAC Methods 985.29 and 991.43 (AACCI Approved Methods 32-05.01 and 32-07.01)[a]

	Method 985.29		Method 991.43[b]		
Sample	HMWDF (%)	SDFS (%)	IDF (%)	SDFP (%)	HMWDF (%)
Cabbage	25.99	1.64	24.90	1.88	26.78
Oat bran	18.22	0.34	13.81	6.91	20.72
Brennan's whole grain brown bread	8.34	0.72	6.49	1.46	7.95
Brennan's wholemeal brown bread	9.95	1.32	9.84	1.74	11.58
Brennan's white bread	4.33	0.95	2.88	1.52	4.40
Raw cauliflower	22.27	1.21	14.07	7.40	21.47
Raw swede	19.65	1.46	14.84	5.43	20.27
Raw potato	7.21	0.23	4.79	3.05	7.84
Weetabix	9.21	1.97	7.62	1.90	9.52
Ryvita dark rye crackers	15.58	4.65	12.39	3.97	16.36
Kellogg's Corn Flakes	2.86	0.01	2.78	0.25	3.03
Carr's water biscuits	4.30	2.32	2.43	1.71	4.14
Flahavan's quick oat porridge	8.74	0.23	6.53	2.44	8.97
Carrot	22.00	0.71	13.98	8.24	22.22
Kellogg's All Bran	25.52	3.40	23.51	2.75	26.26
Whole wheat pasta	9.28	0.80	8.38	2.05	10.43
Broccoli	27.70	0.21	26.79	3.82	30.61
Cooked cauliflower	20.79	0.14	…	…	…
Cooked potato	7.23	0.49	…	…	…
Cooked swede	40.83	0.33	…	…	…
Frosties breakfast cereal	1.68	0.05	…	…	…
Ripe banana	6.44	0.39	…	…	…
Shreddies breakfast cereal	9.40	2.69	…	…	…
Red kidney beans (tinned)	20.00	1.91	…	…	…
Chickpeas (tinned)	19.35	2.11	…	…	…
Sweetcorn (tinned)	13.66	0.29	…	…	…
Cannillini beans	18.59	2.72	…	…	…
Butter beans	17.85	2.32	…	…	…
Garden peas (petit pois)	25.12	1.89	…	…	…

[a] IDF = insoluble DF; SDFP = DF soluble in water but insoluble in 76% aqueous ethanol; and SDFS = DF soluble in water and also soluble in 76% aqueous ethanol.
[b] For AOAC method 991.43, HMWDF is the sum of IDF and SDFP.



bation conditions simulate physiological conditions, which are of considerable importance if new or chemically or physically modified starch-based food ingredients are being introduced.

Analysis of Phosphate Cross-linked Starch (RS_4)

In their article introducing the concept of different types of RS, Englyst et al (1992) identified three classes: RS_1, physically trapped starch as found in coarsely ground or chewed cereals, legumes, and grains; RS_2, RS granules or nongelatinized starch granules that are highly resistant to digestion by α-amylase until gelatinized (e.g., uncooked potato, green banana, and high-amylose starch); and RS_3, retrograded starch polymers, mainly amylose, that are produced when starch is cooled after gelatinization. More recently, phosphate cross-linked starches have been designated RS_4 (Seib and Woo 1997; Brown 2004), and lipid–starch complexes have been referred to as RS_5 (Hasjim et al 2010). Phosphate cross-linked starches have been designated as resistant because a high percentage of this starch measures as DF in AOAC DF methods 985.29 and 991.43. However, if these starches are analyzed by AOAC method 2002.02 (RS) or by DF methods 2009.01 or 2011.25, a much lower resistance to digestion is noted (Table XI). The determined nonresistant starch values for regular maize starch, high-amylose maize starch, and two commercially available RS_4 products are shown in Table III. Samples were either analyzed exactly according to AOAC 2002.02 (RS) or were preheated in incubation buffer at approximately 100°C for 5 min before being cooled and incubated at 37°C with pancreatic α-amylase and AMG. The RS value (determined by difference)

TABLE X

Comparison of Dietary Fiber (DF) Values Obtained Using AOAC Methods 985.29, 2009.01, and 2011.25 (AACCI Approved Methods 32-05.01, 32-45.01, and 32-50.01)[a]

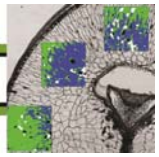
	Method 985.29	Method 2009.01[b]		Method 2011.25[c]		
Sample	HMWDF (%)	HMWDF (%)	SDFS (%)	IDF (%)	SDFP (%)	HMWDF (%)
Cabbage	25.99	28.87	1.12	25.26	2.11	27.37
Oat bran	18.22	18.86	0.54	10.98	8.80	19.78
Brennan's whole grain brown bread	8.34	8.66	1.08	6.80	2.03	8.83
Brennan's wholemeal brown bread	9.95	12.39	1.66	10.19	2.16	12.35
Brennan's white bread	4.33	4.77	0.45	2.95	1.40	4.35
Raw cauliflower	22.27	21.03	0.51	19.02	3.16	22.18
Raw swede	19.65	20.27	1.08	17.41	2.25	19.66
Raw potato	7.21	31.74	0.47	10.84	16.33	27.17
Weetabix	9.21	9.82	2.34	7.38	2.08	9.46
Ryvita dark rye crackers	15.85	16.29	4.70	12.01	4.20	16.21
Kellogg's Corn Flakes	2.86	3.71	0.08	3.11	0.60	3.71
Carr's water biscuits	4.30	4.20	1.53	2.33	1.52	3.85
Flahavan's quick oat porridge	8.74	9.32	0.98	6.12	2.93	9.05
Carrot	22.00	21.81	0.47	17.56	4.90	22.46
Kellogg's All Bran	25.52	26.62	2.45	23.80	2.99	26.79
Whole wheat pasta	9.28	9.90	1.89	8.12	1.60	9.72
Broccoli	27.70	28.09	0.70	29.80	1.67	31.47
Cooked cauliflower	20.79	23.33	0.09	…	…	…
Cooked potato	7.23	10.66	0.57	…	…	…
Cooked swede	40.83	40.41	0.32	…	…	…
Frosties breakfast cereal	1.68	3.10	0.08	…	…	…
Ripe banana	6.44	30.18	0.88	…	…	…
Shreddies breakfast cereal	9.40	10.96	1.97	…	…	…
Red kidney beans (tinned)	20.00	22.80	2.49	…	…	…
Chickpeas (tinned)	19.35	17.98	2.40	…	…	…
Sweetcorn (tinned)	13.66	12.69	0.05	…	…	…
Cannillini beans	18.59	20.53	1.70	…	…	…
Butter beans	17.85	20.16	2.24	…	…	…
Garden peas (petit pois)	25.12	29.08	1.27	…	…	…

[a] IDF = insoluble DF; SDFP = DF soluble in water but insoluble in 76% aqueous ethanol; and SDFS = DF soluble in water and also soluble in 76% aqueous ethanol.
[b] Determined after the additional incubation of this fraction with amyloglucosidase (see Modified Method for SDFS Determination section).
[c] For AOAC method 2011.25, HMWDF is the sum of IDF and SDFP.

TABLE XI

HMW Dietary Fiber (DF) Values Obtained Using AOAC Methods 985.29 and 2009.01 (AACCI Approved Methods 32-05.01 and 32-45.01) for Two Resistant Starch (RS_4) Samples, Fibersym and FiberRite

	Content of HMWDF (%, w/w)			
AOAC Method	Regular Maize Starch	High-Amylose Maize Starch	Fibersym Resistant Starch	FiberRite Resistant Starch
985.29	0.1	25.6	84.0	70.7
2009.01	0.7	49.3	28.7	8.8



for the two RS_4 products is quite low, especially if the product is "cooked" before analysis by AOAC 2002.02. Similar low values were obtained following DF method 2009.01, whereas high values are obtained with the traditional Prosky DF procedure (AOAC 985.29). The low RS values obtained with AOAC methods 2002.02 and 2009.01 were quite unexpected because, in general, higher RS values are obtained with AOAC method 2009.01 compared with 985.29. In attempting to understand this phenomenon, a series of experiments was performed. Initially, the LMW fractions obtained from analyses of FiberRite with AOAC 985.29 and 2009.01 were compared (Fig. 6). Clearly, the major hydrolysis product was glucose, and the relative amount of glucose in each sample can be visually compared by reference to the amount of sorbitol internal standard. To understand this effect more clearly, incubations were performed according to AOAC 2009.01 with the recommended pancreatic α-amylase/AMG mixture but also with pancreatic α-amylase alone and bacterial α-amylase alone at the same level as used in AOAC 985.29, incubated at 37°C. Aliquots were removed at various time intervals and analyzed (Fig. 3). The rate of hydrolysis of FiberRite was similar to that for regular maize starch with all three enzymes. Fibersym was more resistant to hydrolysis but, after 16 h, approximately 65% was hydrolyzed.

To study this issue further, the four starch samples were incubated with thermostable bacterial α-amylase as per AOAC 985.29 but at temperatures of 40–100°C for 1 h. After 1 h, the incubation solutions were filtered, and an aliquot of the filtrate was incubated with AMG to hydrolyze soluble dextrins to glucose, and the glucose was determined with GOPOD reagent (Fig. 4). The degree of hydrolysis was then calculated. With Fibersym, there was little hydrolysis at 40–60°C, substantial hydrolysis at 80°C, and only approximately 12% at 100°C. FiberRite was substantially hydrolyzed at 40–80°C but there was limited hydrolysis at 100°C. It would appear that, at approximately 100°C, the starch granules have gelatinized and expanded dramatically but do not solubilize because of the phosphate cross-linking (something like a balloon expanding) and, thus, impede enzyme access (i.e., there is a physical barrier to access and hydrolysis by α-amylase).

These results indicate that the high DF values previously reported for the RS_4 samples that have been studied in the current work are more a consequence of the assay procedure employed (AOAC methods 985.29 and 991.43) rather than a true resistance to digestion under physiological conditions. If these samples are analyzed under conditions simulating physiological conditions (AOAC 2009.01, pH 6.0, 37°C), much lower DF and RS values are obtained.

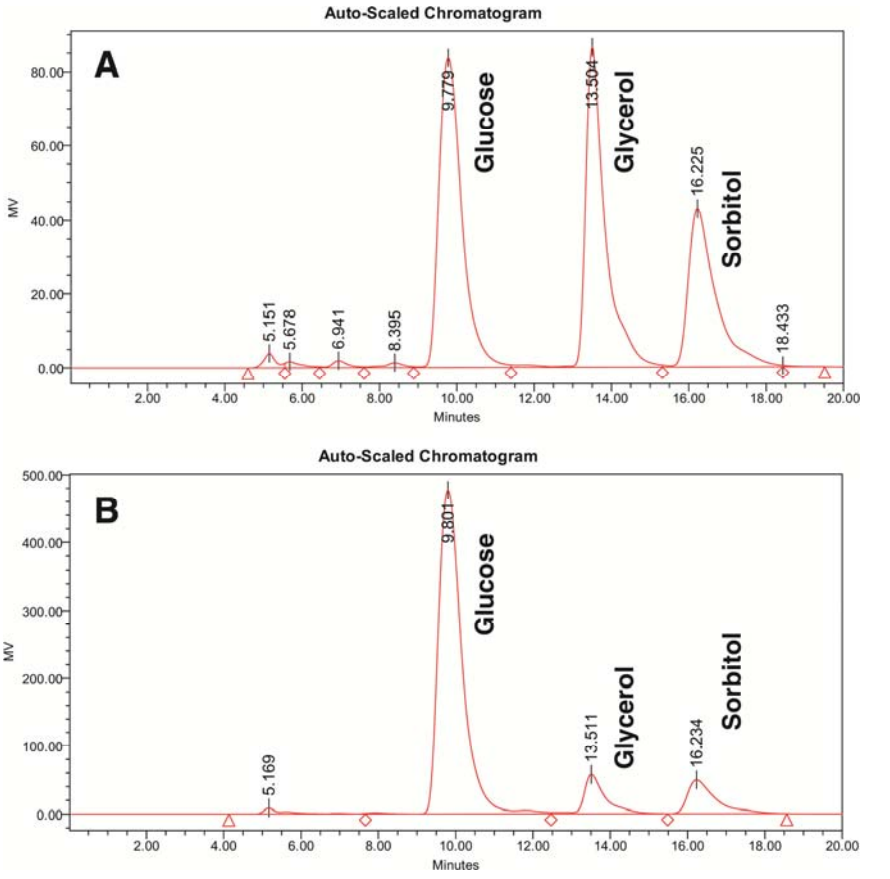
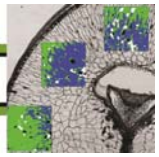


Fig. 6. HPLC analysis of the fraction containing dietary fiber that is soluble in water and also soluble in 76% aqueous ethanol that was produced on hydrolysis of resistant starch RS_4 (FiberRite) in AOAC methods 985.29 (**A**) and 2009.01 (**B**). By comparison of the glucose peak (approximately 9.8) with the sorbitol internal standard peak (approximately 16.2), it is evident that AOAC method 2009.01 results in greater hydrolysis of the RS_4.

Resistant Maltodextrins from Starch Hydrolysis

The analysis by HPLC of the aqueous ethanol soluble fraction (SDFS) from bread samples or pure starches analyzed according to AOAC method 2009.01 reveals a small peak of higher DP material eluting in the range of hepta- to nonasaccharide (Fig. 7). In our original work, we concluded (incorrectly) that this material was either fructooligosaccharides or galactosyl-sucrose oligosaccharides. It has subsequently been shown (Brunt and Sanders 2013) that this material, which represents approximately 0.5–2.0% of the original sample weight (depending on the sample), can be hydrolyzed in the presence of very high concentrations of AMG, showing that it is starch derived. The presence of this residual maltodextrin was unexpected, because the level of AMG used in AOAC method 2009.01 is adequate to give near complete hydrolysis of maltose to glucose, and maltose is hydrolyzed at less than 25% the rate of maltotetraose and higher maltodextrins (McCleary and Anderson 1980). To study this issue further, 6 g of various starch samples were incubated under the conditions used in AOAC 2009.01 and the SDFS fraction chromatographed on Bio-Gel P2 (Fig. 8). The tetrasaccharide, heptasaccharide, and nona-/decasaccharide fractions were recovered and analyzed by NMR and GC-MS.

ESI-MS linkage analysis indicated that the heptasaccharide fraction was mainly composed of terminally linked glucopyranosyl residue (t-Glc), 4-linked glucopyranosyl residue (4-Glc), 6-linked glucopyranosyl residue (6-Glc), and 4,6-linked glucopyranosyl residue (4,6-Glc). The ratio between



t-Glc/6-Glc/4-Glc/4,6-Glc was approximately 3:1:4:1. Small amounts of other Glc residues were also detected in the sample (Table XII). ESI-MS in negative mode indicated the presence of hexose-oligomers with DP7 ([M-H]-1151) as the major component. MS/MS of DP7 hexose-oligomer resulted in a loss of 162 U, which is consistent with a loss of a hexosyl residue. No evidence of a phosphate residue could be detected by ESI-MS analysis. For comparison, ESI-MS of a standard dextran oligomer mixture was run under the same analytical conditions with the sample. [M-H]-1151 is consistent with DP7 hexose-oligomer mass. MS/MS of the 1151 peak also shows a loss of 162 (hexose residue). Lactose-1-phosphate as standard was also analyzed to ensure that ESI-MS conditions were suitable for detection of phosphate-esterified carbohydrates.

On NMR analysis, eight different chemical shifts were clearly observed in the anomeric region of the 1D proton spectrum. Reducing end Glc α- and β-anomers were detected at δ_H 5.20 and 4.62 ppm, respectively (Table XIII). In ^{1}H-^{1}H gCOSY and TOCSY, several spin systems were detected. The majority of the spin systems were similar and appeared to belong to various 4-linked α-Glc residues. From ^{1}H-^{1}H TOCSY, spin system with a δ_H 5.37 ppm anomeric proton belonged to the nonreducing end because it correlated to a δ_H 3.48 ppm characteristic for H-4 of nonreducing end Glc. This signal clearly separates from the other 4-α-Glc ring proton chemical shifts in the TOCSY. However, from combined TOCSY and gHSQC connectivities, it appears that δ_H 5.37 ppm anomeric proton also correlates to protons at δχ 3.96 and 3.71 ppm connected to the carbon at δχ 66.9 ppm. This result indicated downfield shift of the C-6 of the nonreducing Glc residue, suggesting that nonreducing end Glc is 6-linked.

It was much more challenging to find sequences between adjacent internal 4-linked α-Glc residues such as II, III, and IV because of chemical shift similarities and extensive overlap of their ring proton/carbon signals. From multiplicity edited gHSQC, at least three different CH2 pairs (C/H-6) were detected: δχ 61.4, 66.9, and 67.8 ppm, indicating that there were at least two different Glc residues with C-6 shifted downfield (i.e., residues that are 6-linked). TOCSY and gHSQC connectivities revealed that C-6 of δ_H 5.38 ppm anomeric proton was shifted to δχ 67.8 ppm, suggesting that this residue is a 4,6-branched α-Glc. From combined 2D TOCSY, gHSQC correlations, it appeared that C-6 of Glc residues with δ_H 5.30, 4.96, and 4.93 ppm anomeric protons were detected at δχ 61.3 ppm.

It is known that δ_H 5.37 ppm is for H-1 of 6-linked nonreducing end α-Glc and that δ_H 5.38 ppm is for a 4,6-branched α-Glc residue. Anomeric protons at δ_H 4.93 and 4.96 ppm belong to terminal α-Glc1→6 (t-α-Glc1→6) residue according to literature reports. In gHMBC, cross peaks at $\delta_H/\delta\chi$ 4.93/66.9 and 4.96/67.8 ppm suggest that these t-α-Glc residues are linked to position 6 of nonreducing end α-Glc (residue I-6) and to the residue with anomeric proton at δ_H 5.38 ppm (residue III-6) (Fig. 9), respectively.

Linkage between residues I-1* to II-4 and IV-1 to V-4 were confirmed by gHMBC experiment (Table XIII; Fig. 9). Linkage between residues III-1 to IV-4 cannot be unambiguously confirmed because of overlap of chemical shifts. However, linkage of residue I to II and IV to V places the 4,6-branched residue at position III of the oligosaccharide. Collectively, based on 1D and 2D NMR data, the structure depicted in Figure 9 (6^3,6^5-di-α-D-glucosyl maltopentaose) can be proposed for resistant maltoheptaoside (RMD7). NMR data is in good agreement with ESI-MS data, which indicated that RMD7 had MW of 1,152, which corresponds to a heptamer consisting of hexosyl residues (see GC-MS results) (*I-1, II-4 means H-1 of residue I, H-4 of residue II, and so on).

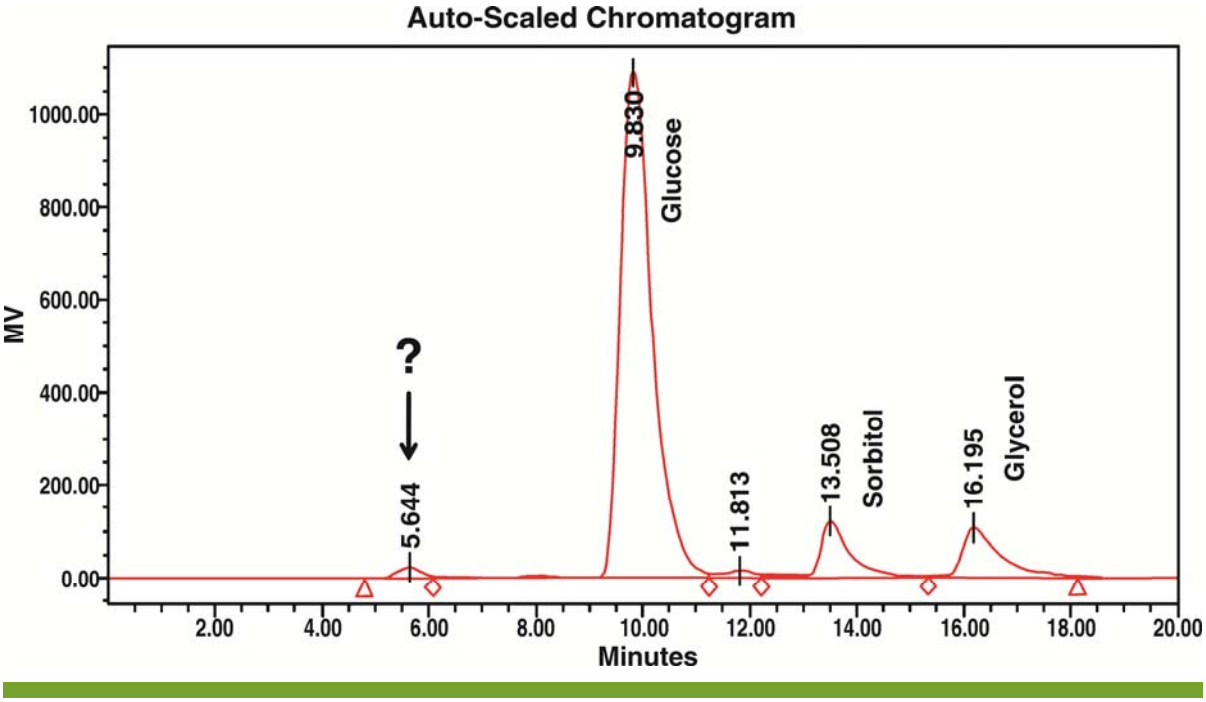


Fig. 7. HPLC analysis of the fraction containing dietary fiber that is soluble in water and also soluble in 76% aqueous ethanol resulting from hydrolysis of regular maize starch in AOAC method 2009.01 (AACCI Approved Method 32-45.01). The peak at retention time 5.644 min is the material of interest, identified as a residual maltodextrin.

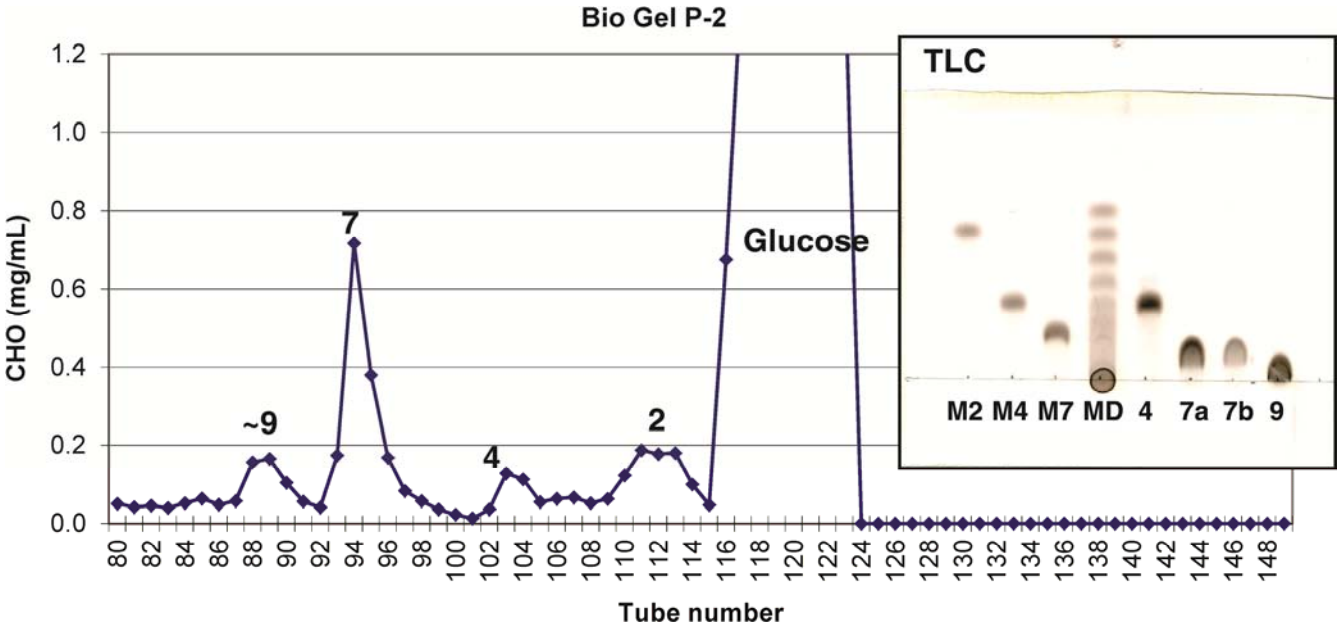


Fig. 8. Bio-Gel P-2 chromatography of the fraction containing dietary fiber that is soluble in water and also soluble in 76% aqueous ethanol resulting from hydrolysis of regular maize starch in AOAC method 2009.01 (AACCI Approved Method 32-45.01). Fractions with a degree of polymerization (DP) of 4, 7, and approximately 9 were collected and analyzed by TLC (insert). In the TLC insert: M2 = maltose; M4 = maltotetraose; M7 = maltoheptaose; MD = maltodextrin mixture; 4 = resistant maltodextrin (DP4); 7a = resistant maltodextrin (DP7) from hydrolysis of regular maize starch; 7b = resistant maltodextrin (DP7) from hydrolysis of whole grain bread; and 9 = resistant maltodextrin (approximately DP9).

TABLE XII
Result of GC/MS Linkage Analysis of the Resistant Maltodextrin Degree of Polymerization 7 in the SDFS Fraction Obtained from Regular Maize Starch Hydrolyzed in AOAC Method 2009.01[a]

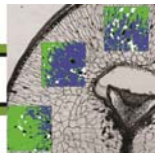
Glycosyl Residue	Peak Area (%)
Terminally linked xylopyranosyl residue	0.050
Terminally linked mannopyranosyl residues	0.541
Terminally linked glucopyranosyl residue	32.012
Terminally linked galactofuranosyl residue	0.080
4-Linked xylopyranosyl residue	0.530
3-Linked glucopyranosyl residue	0.421
2-Linked glucopyranosyl residue	0.514
4-Linked mannopyranosyl residue	0.628
6-Linked mannopyranosyl residue	0.095
6-Linked glucopyranosyl residue	11.905
4-Linked glucopyranosyl residue	41.616
3,4-Linked glucopyranosyl residue	0.374
2,4-Linked glucopyranosyl residue	0.132
4,6-Linked mannopyranosyl residue	0.060
3,6-Linkedglucopyranosyl residue	0.013
4,6-Linked glucopyranosyl residue	10.967
3,4,6-Linked glucopyranosyl residue	0.063

[a] SDFS = dietary fiber soluble in water and also in 76% aqueous ethanol.

Fig. 9. Proposed structure of the resistant maltodextrin (RMD7) produced on hydrolysis of regular maize starch in AOAC method 2009.01 (AACCI Approved Method 32-45.01), as determined by GC-MS and NMR.

The resistant tetrasaccharide was analyzed following similar methodology and found to be 6^3-α-D-glucosyl maltotriose. An inability to separate the nonasaccharide and decasaccharide made structural analysis of these inconclusive.

The resistant heptasaccharide fractions (6^3,6^5-di-α-D-glucosyl maltopentaose) from regular maize starch and from whole grain bread were purified and subjected to hydrolysis by a range of enzymes active on starch and maltodextrins. From the results in Table IV, it is evident that 6^3,6^5-di-α-D-glucosyl maltopentaose is hydrolyzed by AMG but not by an α-glucosidase that readily hydrolyzes linear maltodextrins. However, even hydrolysis by AMG requires approximately 50 times the amount of enzyme required for hydrolysis of maltoheptaose (Table V). AMG at 0.2 U per incubation gives 78% hydrolysis of maltoheptaose but only 1–2% hydrolysis of 6^3,6^5-di-α-D-glucosyl maltopentaose. This heptasaccharide is resistant to hydrolysis by a range of starch- and maltodextrin-degrading enzymes, including α-glucosidase, pancreatic and *A. niger* α-amylases, pullulanase, isoamylase, or mixtures of these enzymes (Table VI). However, the question that remained was "is this heptasaccharide truly resistant to hydrolysis by enzymes of the human small intestine, or is this simply an artifact of the enzymes used in the in vitro assay?" To



answer this question, the enzymes in the brush border lining of the small intestine of pigs were extracted, solubilized, and assayed according to the methods of Dahlqvist (1968) and Dahlqvist and Telenius (1969). Three 30 cm sections of the upper small intestine of a pig were turned inside out, and insoluble maltase-type enzymes were removed by scraping the intestine mucosal membrane with a glass microscope slide, as described by Dahlqvist (1968). The recovered suspension of cellular material was incubated with crystalline papain to solubilize the enzymes. The process is shown in the supplemental video available online. The preparation was devoid of glucose and, thus, could be used directly to analyze for enzyme activity on maltose and on several oligosaccharides containing α-1,6-linked D-glucose; namely, isomaltose, panose, 6^3-α-D-glucosyl maltotriose, and 6^3,6^5-di-α-D-glucosyl maltopentaose. Results obtained are shown in Figure 10. Isomaltose is hydrolyzed at approximately 25% the rate of maltose, consistent with previous publications (Dahlqvist 1968; Dahlqvist and Telenius 1969) and, thus, must be considered to be digestible in the pig (and human) small intestine. Because panose, 6^3-α-D-glucosyl maltotriose, and the 6^3,6^5-di-α-D-glucosyl maltopentaose are hydrolyzed at a rate similar to that of isomaltose, these also must be considered to be readily hydrolyzed and digested in the small intestine. Thus, in the measurement of SDFS from samples containing high starch levels, it is recommended, in agreement with the conclusion of Brunt (2011), that these maltodextrins are removed by hydrolysis with AMG before HPLC.

Hydrolysis and removal of the 6^3,6^5-di-α-D-glucosyl maltopentaose, 6^3-α-D-glucosyl maltotriose, and other maltodextrins containing 6-linked α-D-glucosyl residues are achieved by incubation of the SDFS fraction with AMG at 60°C for 1 h before desalting on resin. The procedure recommended results in no further dilution of this fraction prior to HPLC. This recommendation contrasts to the one by Brunt (2011), in which the sample is diluted a further 10-fold. In our experience, for reproducible results with refractive index determination of HPLC eluates, it is important to work at relatively high sample concentrations. The result of further incubation of the SDFS fraction from rice starch hydrolyzed according to AACCI Approved Method 32-45.01 is shown in Figure 11. The heptasaccharide is hydrolyzed and removed. Similar results were obtained with a range of other starches. This additional incubation with AMG gave no reduction in the amounts of any other NDO analyzed, including fructooligo-

TABLE XIII

NMR Chemical Shift Assignments of the Resistant Maltodextrin Degree of Polymerization 7 in the SDFS Fraction Obtained from Regular Maize Starch Hydrolyzed in AOAC Method 2009.01[a]

Position[b]	Residue	NMR Type	1	2	3	4	5	6	HMBC or NOE
I	→6)-α-Glc-(1→4)	^{1}H	5.37	3.57	3.67	3.48	3.89	3.96/3.71	5.37/78
		^{13}C	100.8	72.5	73.8	70.2	72.1	66.9	…
II	4-α-Glc-(1→4)	^{1}H	5.30	3.60	3.95	3.63	3.87	3.88/3.81	5.3/79.4
		^{13}C	100.7	72.4	74.1	78.0	72.1	61.4	…
III	4,6-α-Glc	^{1}H	5.38	3.69	3.60	3.64	3.88	3.95/3.83	5.38/?
		^{13}C	100.4	72.5	72.4	79.4	72.1	67.8	…
IV	4-α-Glc-(1→4)	^{1}H	5.34	3.63	4.01	3.72*	3.82*	3.96/nd	5.34/74
		^{13}C	100.5	72.2*	71.0	73.8	72.1	nd	…
V	red-α-Glc	^{1}H	5.20	3.53	3.72	3.64	nd	…	…
		^{13}C	92.8	72.3	73.8	74?	nd	nd	…
V	red-β-Glc	^{1}H	4.62	3.24	3.58*	3.63	3.63	3.88/3.75	…
		^{13}C	96.6	74.8	72.4**	78.0	72.2*	61.6	…
VI	t-α-Glc1→6	^{1}H	4.93	3.53	3.72	3.40	nd	3.83/3.77	4.93/66.9
		^{13}C	98.8	72.3	73.8	70.3	…	61.3	…
VII	t-α-Glc1→6	^{1}H	4.96	3.52	3.72	3.42	nd	3.83/nd	4.96/67.8
		^{13}C	99.7	72.3	73.8	70.3	…	61.3	…

[a] SDFS = dietary fiber soluble in water and also in 76% aqueous ethanol; HMBC = heteronuclear multiple bond correlation; and NOE = nuclear Overhauser enhancement. * and ** indicate interchangeable with 78 and 75.3, respectively; nd = not determined.
[b] See Figure 9.

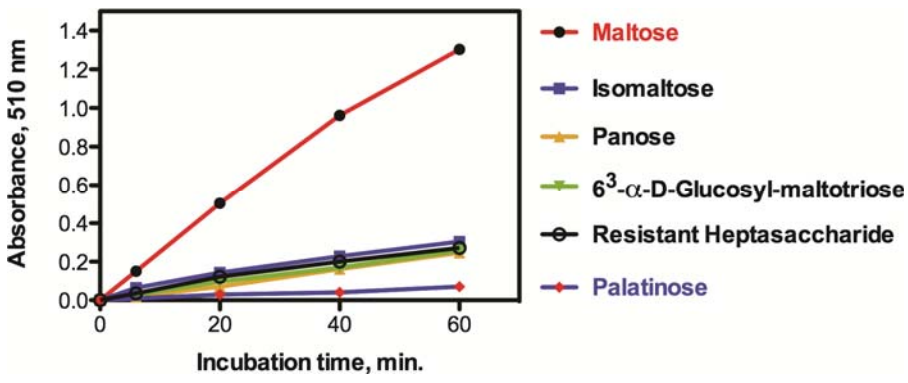


Fig. 10. Hydrolysis of maltose, isomaltose, panose, 6^3-α-D-glucosyl maltotriose, 6^3,6^5-di-α-D-glucosyl maltopentaose, and palatinose by a crude preparation of pig mucosal α-glucosidases.

saccharides, galactooligosaccharides, xylooligosaccharides, Fibersol-2, polydextrose, or stachyose.

Fructooligosaccharides

By definition, DF includes oligosaccharides of DP 3 or greater. The recommended HPLC column to analyze these samples is the Waters Sugar Pak column. However, with this column, a fructosyl-trisaccharde fraction (F3) derived from hydrolyzed chicory inulin elutes after sucrose and, thus, is not included in the fiber content (because the cut-off point is between maltose and maltotriose). Accurate measurement of F3 requires the hydrolysis of sucrose, maltose, and lactose, and this hydrolysis can be achieved enzymically with a mixture of sucrase/maltase and β-galactosidase (Figs. 12 and 13). Hydrolysis of sucrose with sucrase/maltase with no hydrolysis of F3 is shown in Figure 12. Similar results have been obtained for maltose and lactose by using maltase and β-galactosidase (*not shown*). Demonstration of effective hydrolysis of maltose plus lactose or sucrose plus lactose by a mixture of sucrase/maltase plus β-galactosidase is shown in Figure 13. Thus, to correctly measure SDFS by HPLC, the sample is first analyzed by the standard procedure. If there is any shoulder on the disaccharide peak (Fig. 13, sucrose plus lactose), or a separate peak is present at the point at which lactose elutes, an aliquot of the solution as applied to the HPLC column is incubated with sucrase/maltase plus β-galactosidase (E-SUCRBG; Megazyme) and the sample rechromatographed. The amount of F3 is determined by reference to the sorbitol internal standard and added to the already determined SDFS value.

CONCLUSIONS

Several methods are available for the measurement of DF in plant and food products. Of these, only AOAC methods 2009.01 and 2011.25 (AACCI Approved Methods 32-45.01 and 32-50.01) give measurement of all fiber components as defined by Codex Alimentarius. For non-starch-containing samples, AOAC method 2009.01 can be replaced by 985.29, with inclusion of the measurement of SDFS (if required). AOAC method 2009.01 is the recommended reference method in Canada (Health Canada 2012) and is under consideration in other countries. AOAC method 2011.25, which is an extension of 2009.01, is employed when separate determination of insoluble DF and SDFP is required.

Any method designed to measure a mixed group of carbohydrates such as those falling under the Codex definition of DF is

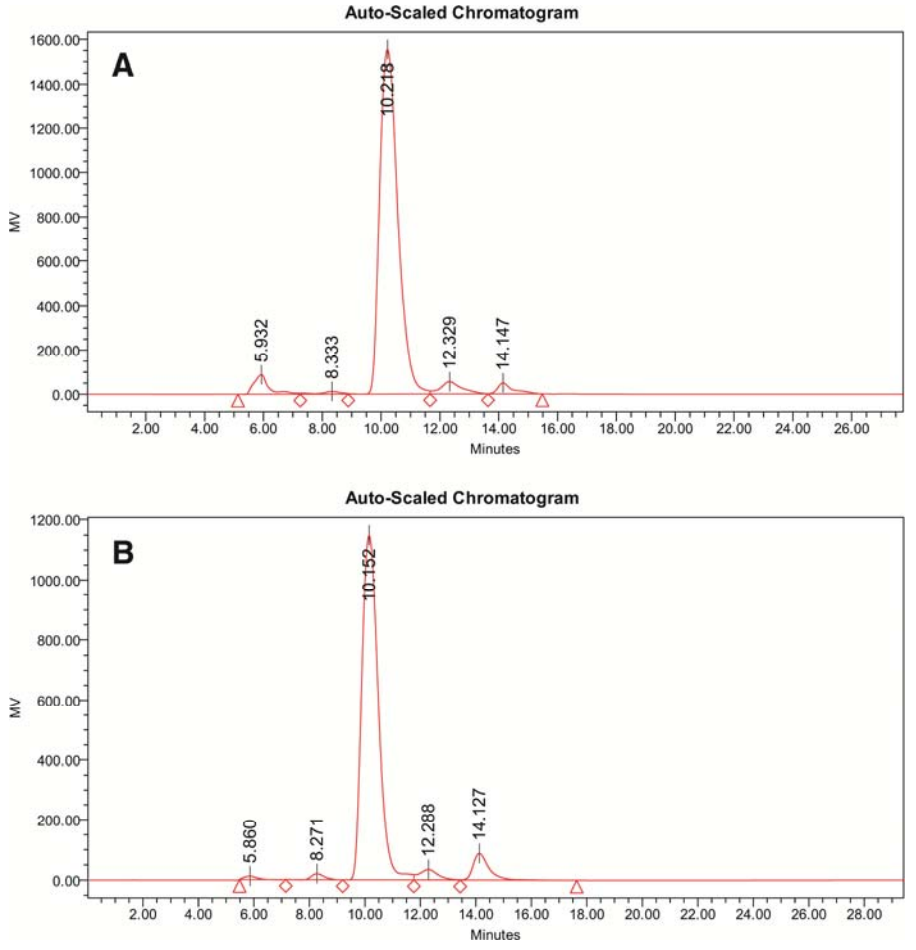


Fig. 11. HPLC analysis of the fraction containing dietary fiber that is soluble in water and also soluble in 76% aqueous ethanol resulting from hydrolysis of rice starch in AACCI Approved Method 32-45.01 (AOAC method 2009.01) before (**A**) and after (**B**) further incubation with amyloglucosidase.

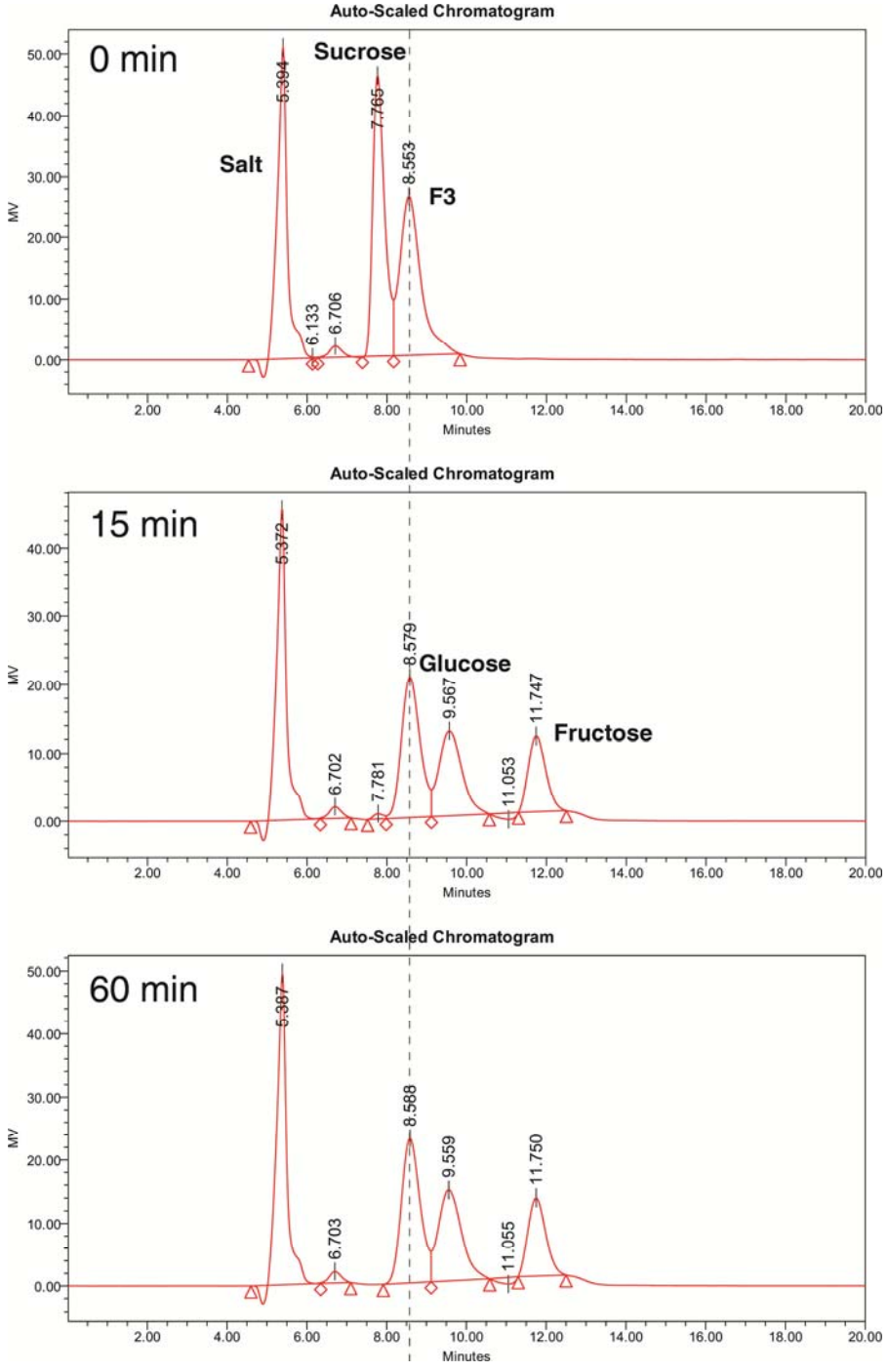


Fig. 12. HPLC analysis of the sugars produced on hydrolysis of sucrose plus fructosyl-β(2-1)-fructosyl-β(2-1)fructose (F3) by sucrase. An aliquot (1.0 mL) of a mixture of F3 (5 mg/mL) and sucrose (5 mg/mL) was incubated with 0.1 mL of sucrase (3 U) plus β-galactosidase (50 U) mixture (Megazyme E-SUCRBG) in 10mM sodium acetate buffer (pH 5.0) at 50°C for 0, 15, and 60 min. The reaction tube was then incubated at 100°C for 5 min. The solution was centrifuged in a microtube at 12,000 rpm for 5 min and the sample directly applied to the HPLC column.

likely to be challenged as new DF ingredients are introduced. This challenge will require further research and, possibly, method modification. In this article, we have discussed modifications required to accurately measure all components of fructooligosaccharides. Values determined for commercial RS_4 materials by AOAC methods 2009.01 and 2011.25 are significantly lower than those obtained with AOAC methods 985.29 and 991.43. Because methods 2009.01 and 2011.25 simulate physiological conditions in the human small intestine, the lower values obtained with these methods are most probably more physiologically significant.

In the analysis of high-starch-containing samples following AACCI Approved Methods 32-45.01 and 32-50.01, some branched maltodextrins resisted hydrolysis and accumulated. More detailed studies on these oligosaccharides, in particular on the heptasaccharide 6^3,6^5-di-α-D-glucosyl-maltopentaose, showed that they were hydrolyzed by the α-glucosidase complex present in the brush border lining of the small intestine of pig (and most likely humans) and, consequently, must be removed to avoid overestimation of the SDFS fraction. This hydrolysis and removal can be achieved by incubation of the SDFS fraction with high levels of AMG. Thus, we recommend that this additional incubation with AMG be included in AOAC methods 2009.01 and 2011.25 (AACCI Approved Methods 32-45.01 and 32-50.01).

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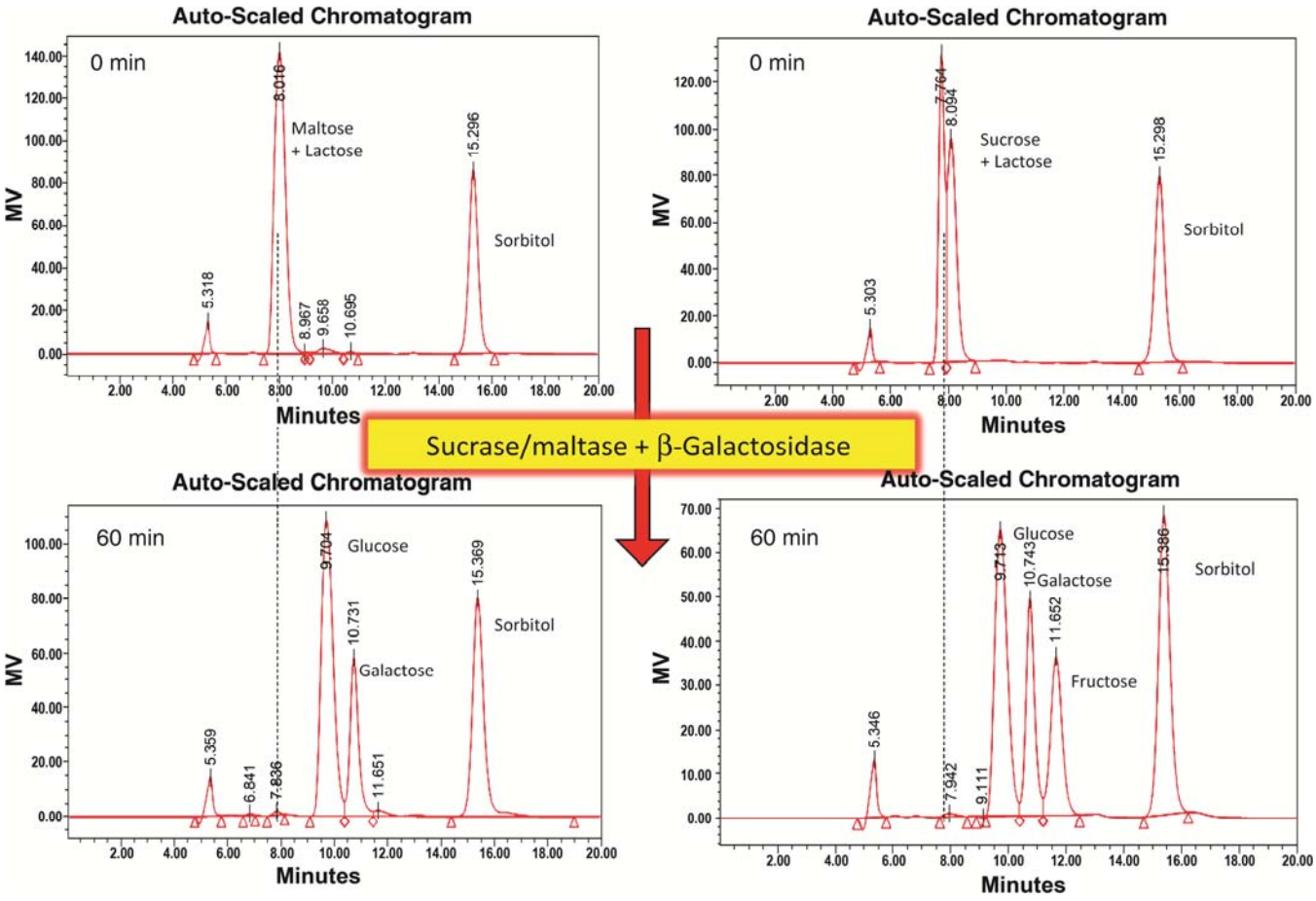
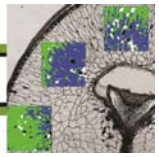


Fig. 13. HPLC analysis of the sugars produced on hydrolysis of a mixture of sucrose plus maltose and of sucrose plus lactose by a mixture of sucrase plus β-galactosidase. An aliquot (1.0 mL) of a mixture of either sucrose (5 mg/mL) plus lactose (5 mg/mL) or maltose (5 mg/mL) plus lactose (5 mg/mL) was incubated with a mixture of 0.1 mL of sucrase (3 U)/α-glucosidase (43 U) plus β-galactosidase (50 U) (Megazyme E-SUCRBG diluted sixfold) in 10mM sodium acetate buffer (pH 5.0) at 50°C for 60 min. The reaction tube was then incubated at 100°C for 5 min. The solution was centrifuged in a microtube at 12,000 rpm for 5 min and the sample directly applied to the HPLC column.



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