

# Development of an Integrated Total Dietary Fiber Method Consistent with the Codex Alimentarius Definition

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## History

Interest in dietary fiber is a consequence of the belief that dietary fiber contributes positively to the health/quality of life of the consumer. The physiological effects of dietary fiber are what make it of interest to the consumer, food nutritionists, and regulators. Because dietary fiber is a multicomponent mixture, it is essential that there is a clear definition and a methodology to allow measurement of the defined components.

Over the past thousands of years, the human diet has changed from one based on a wide range of coarse plant materials with little animal products to one in which the plant components are limited to few plant products (which are highly milled and processed) and a larger portion of animal products. The need for coarse foods of plant origin to combat constipation can be traced back to Hippocrates in the 4th century BC (5), who commented on the laxative action of the outer layers of cereal grains.

A great deal of debate has continued on the definition of dietary fiber over the past 60 years, together with discussions on methods for measuring this dietary fiber. Disagreement on whether to define dietary fiber by its physiological attributes or by its chemical composition continues even today. In 1953, Hipsley (10) defined dietary fiber as being composed of cellulose, hemicellulose, and lignin. This was broadened by Trowell in 1976 (22) to include soluble substances (non-cell wall-derived materials), such as pectins, gums, and mucilages.

At an AOAC International Spring Workshop in Ottawa in 1981 (4), the scientists present, who included the leaders in the field at that time (Asp, Southgate, Baker, Van Soest, and Heckman), concluded that two methods for the determination of total dietary fiber (TDF) in foods should be developed, including: a) a rapid enzymatic gravimetric method based on the procedure developed by Asp, et al. (4), Furda, et al. (9), and Schweizer and Wursch (19); and b) a more comprehensive method, such as a modification of the methods of Southgate (20) or Theander and Aman (21), was needed to determine the individual dietary fiber components. In the rapid enzymatic-gravimetric method, the sum of soluble and insoluble polysaccharides and lignin would be defined and measured as a unit (AACC Intl. Method 32-05.01) (1). With slightly modified methodology, soluble and insoluble dietary fiber could be measured separately (AOAC Official Methods 993.19, 991.42, and 991.43) (3) (Table I).

The basic definition of dietary fiber that was accepted by scientists throughout the world was that proposed by Trowell, et al. in 1976 (22). It stated that “dietary fibre is composed of the remnants of plant cells resistant to hydrolysis by human alimentary enzymes and that it included all indigestible polysaccharides

(celluloses, hemicelluloses, oligosaccharides, pectins, gums) plus waxes and lignin.” A working group headed by Leon Prosky set about turning this definition into a method that could pass a collaborative study and be adopted by AOAC International as an approved method. The resulting method, based on the use of thermostable  $\alpha$ -amylase, amyloglucosidase (AMG), and protease to hydrolyze and remove starch and protein, passed an AOAC International interlaboratory evaluation and subsequently was accepted as AACC Intl. Method 32-05.01/AOAC Official Method 985.29 (i.e., the Prosky “gold standard” method) (18). Subsequent modifications allowed the measurement of soluble and insoluble dietary fiber (AACC Intl. Method 32-07.01/AOAC Official Method 991.43) (12). The buffer system used was subsequently changed from phosphate to MES-Tris (AOAC Official Method 991.43) (3,12). The main reason for the change of buffer was that at that time, thermostable  $\alpha$ -amylase preparations contained high levels of calcium to stabilize the enzyme. This calcium reacted with the phosphate to form calcium phosphate that precipitated from solution and contributed to ash levels. This variable precipitation added to the variability of results. In more recent years, thermostable  $\alpha$ -amylase preparations that are less dependent on calcium levels have become available, so the problems related to calcium phosphate precipitation no longer exist. From the outset, it was known that protein was not completely hydrolyzed by the protease employed, so samples were analyzed in duplicate; one of the residues being analysed for ash and the other for residual protein and these weights are subtracted from the average of the residue weights for the sample. The fact that all of the starch was not hydrolyzed was ignored. The presence of a starch fraction resistant to enzymic hydrolysis was first recognized by Englyst, et al. in 1982 (8) during their research on the measurement of nonstarch polysaccharides.

**Table I. Equivalent AACC Intl. and AOAC International methods for measurement of dietary fiber and dietary fiber components**

Analyte	AACC Intl. Approved Method	AOAC Official Method
Total dietary fiber (Prosky)	32-05.01	985.29
Soluble dietary fiber	-	991.42
Insoluble dietary fiber	-	993.19
Total dietary fiber (Lee)	32-07.01	-
Total soluble and insoluble dietary fiber (Lee)	-	991.43
Total dietary fiber (as neutral sugars, uronic acids, and klason lignin)	-	994.13
Mixed-linkage $\beta$ -glucan	32-22.01	992.28
	32-23.01	995.16
Fructo-oligosaccharides	32-31.01	997.08
and fructan	32-32.01	999.03
Resistant starch	32-40.01	2002.02
Resistant maltodextrins	32-41.01	2001.03
Galacto-oligosaccharides	32-33.01	2001.02
Polydextrose	32-28.01	2000.11

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The Trowell (22) definition of dietary fiber and the matching methods served the scientific community well until the early 1990s when it was realized that nondigestible oligosaccharides (NDO)—which do not precipitate in the 78% ethanol used in the classical AACC Intl./AOAC Approved Methods—and resistant starch (RS) possess many of the characteristics commonly associated with dietary fiber and is resistant to hydrolysis by the enzymes in the human small intestine. At that time, a committee of scientists appointed by the Executive Committee of AACC Intl. were holding meetings, with invitations extended to the scientific community at large to participate in order to determine if the definition of dietary fiber should remain as originally proposed, or due to the accumulation of scientific data, be changed or altered in any way to accommodate these new compounds. An international survey of scientists in 1993 showed that 65% of the respondents favored the inclusion of NDO in dietary fiber and 80% favored the inclusion of RS. This led to the development of specific fiber methods for the measurement of fructo-oligosaccharides (AACC Intl. Methods 32-31.01 and 32-32.01; AOAC Official Methods 997.08 and 999.03) (1,3), galacto-oligosaccharides (AACC Method 32-33.01; AOAC Official Method 2001.02) (10,11), resistant maltodextrins (AACC Method 32-41.01; AOAC Official Method 2001.03) (1,3), Polydextrose<sup>R</sup> (AACC Method 32-28.01; AOAC Official Method 2000.11) (1,3), and resistant starch (AACC Intl. Method 32-40.01; AOAC Official Method 2002.02) (1,3,15). However, one problem remained; a portion of some components that are measured by the specific method was also measured by the total dietary fiber methods (Fig. 1), i.e., there was some double counting.

In 1998, AACC Intl. (formerly the American Association of Cereal Chemists) began a critical review of the current state of dietary fiber science, including the consideration of the state of the dietary fiber definition. Over the course of the next year, the committee held three workshops and provided an international website, available to all web users worldwide, to receive comments. All interested parties were provided with an additional opportunity for comment. After due deliberation, an updated definition of dietary fiber was delivered to the AACC Intl. Board of Directors for adoption in early 2000 and published (2) namely: “Dietary fiber is the edible parts of plants or analogous carbohydrates that are resistant to digestion and absorption in the human small intestine with complete or partial fermentation in the large intestine. Dietary fiber includes polysaccharides, oligosaccharides, lignin, and associated plant substances. Dietary

fibers promote beneficial physiological effects including laxation, and/or blood cholesterol attenuation, and/or blood glucose attenuation.”

Several definitions of dietary fiber have appeared over the past five years. The Food Nutrition Board (FNB) of the Institute of Medicine of the National Academies (U.S.A) (11) defined dietary fiber in 2001 as follows: “Dietary fiber consists of nondigestible carbohydrates and lignin that are intrinsic and intact in plants. Added fiber consists of isolated, nondigestible carbohydrates that have beneficial physiological effects in humans. Total fiber is the sum of dietary fiber and added fiber.”

The need for a clear definition of dietary fiber to support nutrition claims has been an agenda item for the Codex Alimentarius Commission since 1992. This effort has been led by the Codex Committee on Nutrition and Foods for Special Dietary Uses (CCNFSDU). The definition of dietary fiber that arose from the 27th session of CCNFSDU (ALINORM 06/29/26), in Bonn, Germany, November 21–25, 2005, (6) was similar in many respects to that proposed by AACC Intl., but with no reference to physiological effects, namely:

“Dietary fibre means carbohydrate polymers with a degree of polymerization (DP) not lower than 3 which are neither digested nor absorbed in the small intestine. A degree of polymerization not lower than 3 is intended to exclude mono- and disaccharides. It is not intended to reflect the average DP of the mixture. Dietary fibre consists of one or more of:

- Edible carbohydrate polymers naturally occurring in the food as consumed;
- Carbohydrate polymers which have been obtained from raw materials by physical, enzymatic or chemical means;
- Synthetic carbohydrate polymers.”

At the 2008 meeting of CCNFSDU in Cape Town, South Africa (7), a consensus decision on the definition of dietary fiber was achieved:

“Dietary fibre means carbohydrate polymers<sup>2</sup> with ten or more monomeric units<sup>3</sup>, which are not hydrolyzed by the endogenous enzymes in the human 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 raw materials 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.
- 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.”

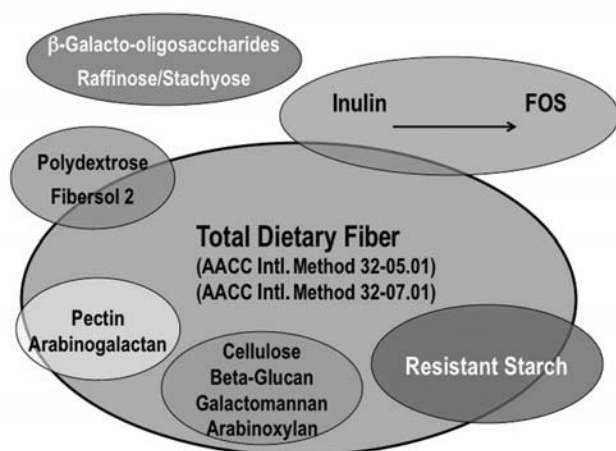


Fig. 1. Components measured, and not measured, by AACC Intl. Methods 32-05.01 and 32-07.01 (1).

<sup>2</sup> When derived from a plant origin, dietary fiber may include fractions of lignin and/or other compounds when associated with the 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 are often extracted with the polysaccharides in AOAC 991.43 method. These substances are included in the definition of fiber insofar as they are actually associated with the poly- or oligo-saccharidic fraction of fibre. However, when extracted or even re-introduced in to a food containing non digestible polysaccharides, they cannot be defined as dietary fibre. When combined with polysaccharides, these associated substances may provide additional beneficial effects (pending adoption of Section on Methods of Analysis and Sampling).

<sup>3</sup> Decision on whether to include carbohydrates from 3 to 9 monomeric units should be left to national authorities.

## Development of an Integrated TDF Method

Within the Dietary Fiber and Other Carbohydrates Technical Committee of AACC Intl., the need for an all-encompassing method for the measurement of dietary fiber was realized, discussed, and worked on for the past five years. Clearly, such a method must accurately measure RS and allow for the measurement of NDO. A method that meets these requirements, published in 2007 (20), is outlined in Figure 2. The aim of the method was to give a measurement of RS in line with *in vivo* results and to recover NDO intact. The key steps in the method are: a) incubation of sample with pancreatic  $\alpha$ -amylase and amyloglucosidase to effect hydrolysis of non-RS. It is essential that the enzymes employed are sufficiently pure as to cause no depolymerization and loss of high molecular weight soluble dietary fiber (HMWSDF) and that there is no degradation of NDO (low molecular weight soluble dietary fiber [LMWSDF]); b) the denaturation of protein by incubation of a sample at 100°C. This step is necessary for uncooked samples; otherwise, the protein will not be hydrolyzed and depolymerized by protease. Adjusting the pH to 8 before heating at 100°C prevents AMG action during the heating process; pancreatic  $\alpha$ -amylase is inactivated well below the temperature required for gelatinization of RS (>60°C); c) the adjustment of pH to ~4.3 and precipitation of HMWSDF with 4 volumes of ethanol. Charged polysaccharides can differ in how they precipitate at different pH values, so the pH value chosen for this step of the current method is the same as that used in AOAC Official Method 985.29 (3); d) the recovery of precipitated HMWSDF and insoluble dietary fiber (IDF) on a sintered glass crucible, washing with ethanol and acetone, and drying to yield high molecular weight dietary fiber (HMWDF). After adjusting the pH of the reaction solution to 4.3, an aliquot (1.0 mL) of solution can be removed for the determination of available carbohydrates (if this value is required) and an aliquot (1 mL) of D-sorbitol internal standard (100 mg/mL) is added; and d) the concentration of filtrate, desalting, reconcentration, and HPLC analysis of LMWSDF.

## Interlaboratory Evaluation of the Integrated TDF Method

The method described above was subjected to interlaboratory evaluation under the auspices of AACC Intl. and AOAC International, with representation of laboratories that fall under the International Association of Cereal Science and Technology (ICC). Eighteen laboratories participated with 16 laboratories returning

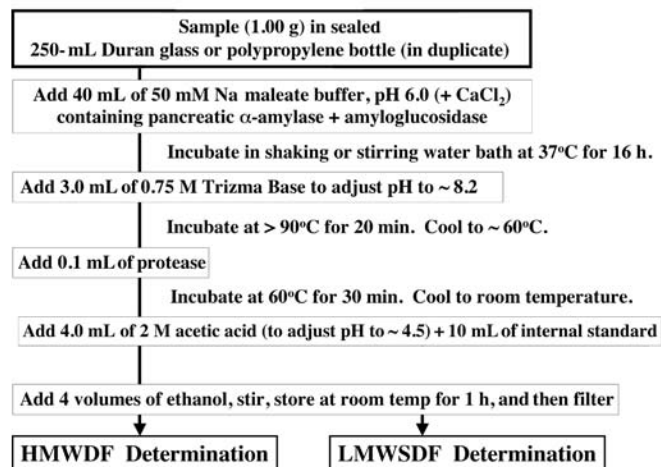


Fig. 2. Principle of the Integrated (Codex compliant) Total Dietary Fiber Assay Procedure.

valid assay data for 16 test portions (eight blind duplicates) consisting of samples with a range of traditional dietary fiber, RS, and NDO. The dietary fiber content of the eight test pairs ranged from 11.57 to 47.83%. Digestion of samples under the conditions of AACC Intl. Method 32-40.01 (1) followed by the isolation and gravimetric procedures of AACC Intl. Methods 32-05.01 and 32-07.01 (1) results in the quantitation of HMWDF. The filtrate from the quantitation of HMWDF is concentrated, deionized, concentrated again, and analyzed by HPLC to determine LMWSDF, i.e., all nondigestible oligosaccharides of DP 3 or higher. TDF is calculated as the sum of HMWDF and LMWSDF. Repeatability standard deviations ( $s_r$ ) ranged from 0.41 to 1.43, and reproducibility standard deviations ( $s_R$ ) ranged from 1.18 to 5.44 (14). This is comparable to other official dietary fiber methods. At this stage, the method has been accepted as AACC Intl. Method 32-45.01 and AOAC Official First Action Method 2009.01 (1,3).



Fig. 3. Picture showing a 2mag Mixdrive 15 submersible magnetic stirrer in a custom made water bath. This allows stirring of 15 samples at controlled speed and 37°C.

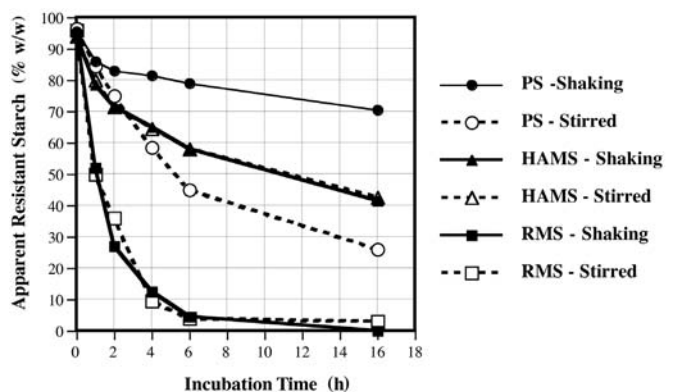


Fig. 4. Rate of hydrolysis (and apparent resistant starch level) of regular maize starch (RMS), high amylose maize starch (HAMS), and native potato starch (PS) on incubation at 37°C with  $\alpha$ -amylase/AMG in Duran bottles either shaken in rotary motion at 150 rpm in a Grant OLS 200 shaking incubation bath or stirred at 170 rpm with  $\alpha$ -amylase/AMG in Duran bottles in a water bath at 37°C on a 2mag Mixdrive 15 submersible magnetic stirrer (Fig. 3). (In this case, the stirrer bars were added to the Duran bottles rather than being suspended.) Note that the values for HAMS and RMS are the same with both mixing and stirring. This was found for all samples studied except native potato starch, which has a fragile granule structure.

### Further Development of the Integrated TDF Method

During the process of performing the interlaboratory study, certain issues became evident and various potential improvements of the method became apparent. These can be discussed under several headings.

#### Incubation with $\alpha$ -amylase Plus AMG

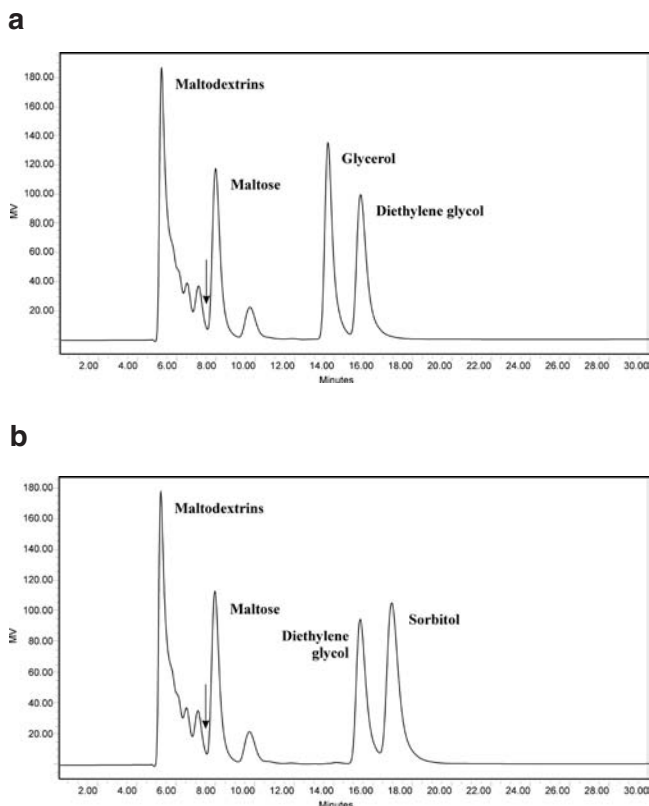
In AOAC Official Method 2009.01 (3), incubations with pancreatic  $\alpha$ -amylase plus AMG are performed in a shaking water bath in rotary motion. Not all laboratories have such equipment, so the possibility of using a setup employing magnetic stirring with either suspended magnetic stirring bars (no grinding of sample between the stirrer bar and the bottom of the Duran bottle; Fig. 3) or with stirrer bars added directly into the Duran bottles was evaluated. Apparent resistant starch values obtained throughout the incubations for regular maize starch, high amylose maize starch, and native potato starch are shown in Figure 4. The only sample for which different values were obtained with the different mixing methods was native potato starch. Native potato starch does show a high resistant starch value in ileostomy studies, but this is a fragile starch and thus cannot be processed by either heating or mixing without losing a high proportion of the resistant starch content. All other resistant starch containing samples (including kidney beans and green banana) gave the same values for the three mixing methods. We thus conclude that any of the three mixing/stirring procedures that were evaluated are suitable.

#### Incubation to Denature Protein

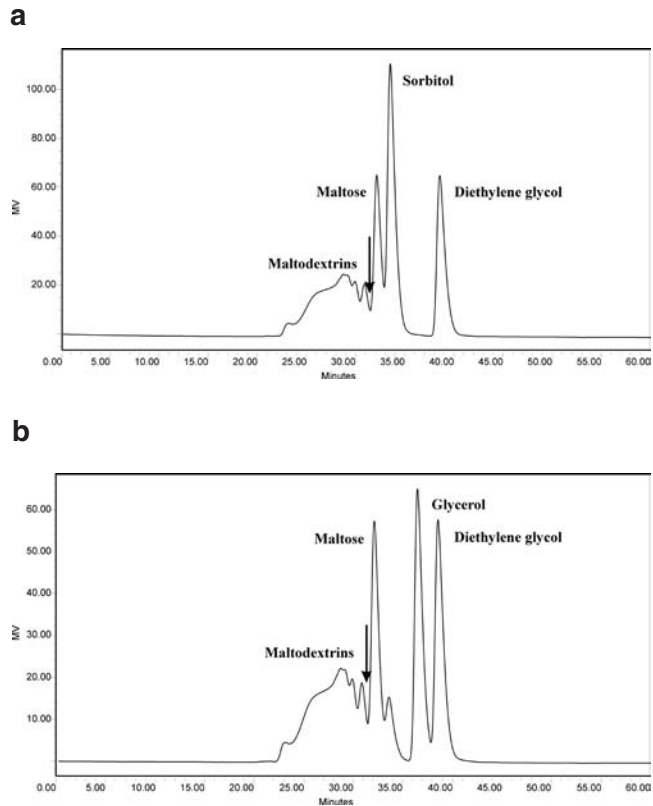
Samples that have been cooked as part of their preparation for human consumption (e.g., bread, cooked pasta, or rice), may not require heating to  $\sim 100^\circ\text{C}$  to denature protein, as the protein is already denatured. However, if the incubation at  $\sim 100^\circ\text{C}$  is deleted, it is essential to show there is no hydrolysis of partially solubilized resistant starch by AMG during the protease incubation step for 30 min at  $60^\circ\text{C}$  and pH 8. More work is required in this area.

#### Internal Standard

In the original method, published in 2007 (20), D-sorbitol was chosen as the internal standard using a Waters Sugar Pak HPLC column and matching precolumn. D-Sorbitol chromatographs separately from sugars, oligosaccharides, and glycerol. However, some laboratories prefer to use the TSK-Gel columns (two TSK-Gel 30 cm  $\times$  7.8 mm columns connected in series; G2500PWXL, Sigma Aldrich, Dublin, Ireland) as used in AOAC Official Method 2001.03. In AOAC Official Method 2001.03, glycerol is used as the internal standard, but this is not suitable with the current method as two of the enzymes employed contain glycerol as a stabilizer. D-Sorbitol is not suitable as it chromatographs and elutes with D-glucose (3). Clearly, an internal standard that can be used with both chromatographic systems is desirable (3). Work within Megazyme and Matsutani Chemical Company identified 1,5-pentanediol, diethylene glycol, and triethylene glycol as suitable. None of these compounds is lost on rotary



**Fig. 5.** Chromatography of a mixture of maltodextrins, maltose, diethylene glycol, plus either a) glycerol; or b) D-sorbitol on a Waters Sugar-Pak (6.5  $\times$  300 mm); solvent: distilled water containing Ca-EDTA (50 mg/L); flow rate: 0.5 mL/min; temperature:  $90^\circ\text{C}$ . The arrows show demarcation between DP 2 (maltose) and DP 3 (higher maltodextrins).



**Fig. 6.** Chromatography of a mixture of maltodextrins, maltose, diethylene glycol plus either (a) D-sorbitol, or (b) glycerol on two TSK gel filtration columns (G2500PWXL) in series. Solvent: distilled water; flow rate: 0.5 mL/min; temperature:  $80^\circ\text{C}$ . The arrows show demarcation between DP 2 (maltose) and DP 3 (higher maltodextrins).

evaporation or through binding to the desalting resins that are employed. However, of the three, diethylene glycol (recommended by Kazuhiro Okuma, Matsutani Chemical Industry Company, Japan) is considered to be the best option as it chromatographs separately from sugars, oligosaccharides, glycerol, and D-sorbitol with both chromatographic systems (Figs. 5 and 6).

#### *In-Line Desalting*

Following the concentration of the ethanolic filtrate in the standard method, samples are desalted through resins and re-concentrated before applying to the HPLC column. An alternative is to concentrate the ethanolic filtrate to dryness and redissolve in 10 mL of distilled water, filter, and then apply a sample to the HPLC column via a de-ashing column (16). The use of such a column significantly reduces time associated with sample handling, but the de-ashing columns are expensive and this must be balanced against time saving.

#### *Effect of Microorganisms and Enzymes on Samples Being Analyzed*

The possibility that the method could be compromised by either enzymes or microorganisms in the samples being analyzed has been raised by scientists reviewing the procedure. The presence of sodium azide in the incubation buffer removes any likely problems with microbial contamination or proliferation. Most samples being analyzed will not contain enzyme activity (e.g., cooked food samples and most milled grain and fiber components). However, some samples do contain enzyme activity and this could reduce fiber content, e.g., malt samples, which contain residual levels of  $\beta$ -glucan plus high activity of  $\beta$ -glucanase. Such samples have not been studied in detail; however, a likely solution to this problem would involve a pre-incubation of the sample in ethanol (5 or 10 mL at  $\sim 100^\circ\text{C}$ ) before the addition of  $\alpha$ -amylase plus AMG enzymes in the buffer. This potential problem will be studied over the coming months.

#### **Conclusions and Further Action**

A method has been developed for analyzing TDF in line with the new Codex Alimentarius definition. The procedure captures all resistant starch and the aqueous ethanol filtrate is analyzed for NDO. A sample can be removed for determination of available carbohydrates (measured as D-glucose and D-fructose). This procedure has been successfully subjected to interlaboratory evaluation and the method accepted as AACC Intl. Method 32-45.01 and AOAC Official First Action Method 2009.01. The evaluation of the interlaboratory results by AACC Intl. is underway.

A second interlaboratory study is planned in which a broader range of samples will be analyzed and modifications described above will be introduced. The effect of cooking and not cooking on the determined RS of particular precooked samples will also be evaluated.

#### **Editor's Note**

As the January-February issue of *Cereal Foods World* went to press, the AACC Intl. Approved Methods Technical Committee was reviewing this method for inclusion in the online *AACC International Approved Methods of Analysis, 11th Edition*. If approved, the method is expected to post online by February 2010.

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