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Article *in* Journal of AOAC International · January 2010

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Determination of Total Dietary Fiber (CODEX Definition) by Enzymatic-Gravimetric Method and Liquid Chromatography: Collaborative Study

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ABSTRACT

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A method for the determination of total dietary fiber (TDF), as defined by the CODEX Alimentarius, was validated in foods. Based upon the principals of AOAC Official Methods[i] 985.29, 991.43, 2001.03, and 2002.02, the method quantitates high and low molecular weight dietary fiber. McCleary[ii], in 2007 described a method of extended enzymatic digestion at 37°C to simulate human intestinal digestion followed by gravimetric isolation and quantitation of high molecular weight dietary fiber (HMWDF) and the use of liquid chromatography (LC) to quantitate low molecular weight soluble dietary fiber (LMWSDF). The method thus quantitates the complete range of dietary fiber components from resistant starch (by utilizing the digestion conditions of AOAC 2002.02) to digestion resistant oligosaccharides (by incorporating the deionization and LC procedures of AOAC 2001.03). The method was evaluated through an AOACI collaborative study. Eighteen laboratories participated with 16 laboratories returning valid assay data for 16 test portions (8 blind duplicates) consisting of samples with a range of traditional dietary fiber, resistant starch, and non digestible oligosaccharides. The dietary fiber content of the 8 test pairs ranged from 11.57 to 47.83 %. Digestion of samples under the conditions of AOACI 2002.02 followed by the isolation and gravimetric procedures of AOACI 985.29 and 991.43 results in quantitation of high molecular weight dietary fiber (HMWDF). The filtrate from the quantitation of HMWDF is concentrated, deionized, concentrated again, and analyzed by LC to determine the low molecular weight soluble dietary fiber (LMWSDF) i.e. all non digestible oligosaccharides of DP = 3 or higher. Total dietary fiber 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. This is comparable to other Official dietary fiber methods. This method is recommended for adoption as Official First Action.

INTRODUCTION

Dramatic increases in the utilization of fiber analysis in fiber research and the marketing, research and development of food products have accompanied increases in public awareness of the health benefits of high fiber foods over the past several decades. As researchers have discovered and elucidated additional dietary fiber sources, not only has there been a need to update the definition of dietary fiber, but also to update the methodologies that support the definition. AOAC International has been a leader in providing Official Methods of Analysis consistent with the state of dietary fiber science[iii]. In the 1970's, Trowell and fellow dietary fiber researchers[iv, v, vi, vii] published a definition later adopted as consensus by AOAC International following an international survey by Prosky[viii] in the late 1970's.

The Trowell et al. definition:

Dietary fiber consists of the plant polysaccharides and lignin which are resistant to hydrolysis by digestive enzymes of man. This definition defines a macro constituent of foods which includes cellulose, hemicellulose, lignin, gums, modified celluloses, mucilages, oligosaccharides, and pectins and associated minor substances such as waxes, cutin, and suberin.

As the science of dietary fiber has advanced, the Official Methodology (i.e. 985.29 and its extensions (991.42, 993.19) [ix] and methods which give equivalent results, (991.43[x], 992.16, 993.21, and 994.13) which adequately quantitated the known dietary fiber food fractions at the time of the Trowell et al. definition, have become insufficient to quantitate other dietary fiber components which were unknown at the time of adoption of those official methods. Advances in understanding the complexity of dietary fiber, and that food components such as resistant starch, fructans, polydextrose, and resistant maltodextrins are part of dietary fiber in the diet led to the development and adoption of Official Methods 997.08 and 999.03,

2000.11, 2001.02, 2001.03 and 2002.02 (for the quantitation of fructans, polydextrose, *trans*-galactooligosaccharides, resistant maltodextrins, and resistant starch respectively). While these methods perform well for their individual components, obtaining a total dietary fiber value involves performing multiple analyses and mathematical summations, and in some cases carrying out special procedures to assure against "double counting" of a particular dietary fiber fraction[xi].

Recently, international authorities on dietary fiber definition, working through the CODEX Committee on Nutrition and Foods for Special Dietary Uses (CCFNSDU), have updated the terminology of the dietary fiber definition[xii].

CODEX defines dietary fiber as carbohydrate polymers[a] with ten or more monomeric units[b], 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,**
- **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 fibre 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. These substances are included in the definition of fibre 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).

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

With the possible exception of the synthetic carbohydrate polymers, the food components included in the CODEX definition match those of the more broadly stated Trowell et al. [vii] definition. Therefore the currently adopted Official Methods can be readily applied, although the same issues of extra expense for carrying out multiple assays and the need for mathematical summations and carrying out of special procedures to avoid double counting apply. Obviously, a single method that quantitates the entirety of components included in the Trowell and the CODEX definitions is desired. In as much as the CODEX

definition includes resistant oligosaccharides of the size of 3 monomeric units and larger (as had previous proposals agreed upon within the CODEX committee), a relevant method must meet that criteria. If a demarcation between DP 9 and DP 10 is necessary, should that national authority option be pursued, methodology will have to be worked out. This may prove to be a difficult task, According to Ku et al.[xiii] some carbohydrate polymers of DP<10 may remain in the alcoholic precipitate while for other carbohydrates, polymers of length far greater than DP 10 are not precipitated. Therefore there is currently no clear cut approach to quantitation at a demarcation point between DP 9 and DP 10.

McCleary [ii] in 2007 described a method that incorporates key features of Official Methods 985.29, 991.43, 2001.03 and 2002.02[xiv],[xv] to provide an assay that quantitates the food fraction as delineated by CODEX Alimentarius (CCNFSDU). This methodology addresses the issues expressed above, and provides a method that is based on methodologies accepted by industries and authorities worldwide. One remaining concern with the inclusive method is that it takes two days to carry samples through the entire process of analysis. The all inclusive nature of the most recent methods has increased the time relative to 985.29 and 991.43. However, this is deemed necessary to assure quantitation of all fiber fractions, and consumes less time than carrying out several assays separately so the results can be mathematically combined.

AOAC Official Method 2009.xx

Total Dietary Fiber in Foods

Enzymatic-Gravimetric-Liquid Chromatography Method

(Applicable to plant material, foods, and food ingredients consistent with CODEX Definition 2008 (ALINORM 09/32/26), including naturally occurring, isolated, modified, and synthetic polymers meeting that definition).

A. Principle

A method is described for the measurement of total dietary fiber, including resistant starch (RS) and low molecular weight non-digestible oligosaccharides (LMWNDO) of DP $\geq$3 (Figure 1). This method combines the key attributes of AOAC Official Methods of Analysis 985.29, 991.43, 2001.03, and 2002.02 (Figure 2). Duplicate test portions are incubated with pancreatic α-amylase and amyloglucosidase (AMG) for 16 hr at 37^{o}C in sealed 250 mL bottles in a shaking water bath while mixing with sufficient vigor to maintain continuous suspension. During this step, non-resistant starch is solubilized and hydrolyzed to glucose and maltose by the combined action of the two enzymes. The reaction is terminated by pH adjustment and temporary heating. Protein in the sample is digested with protease. For the measurement of high molecular weight dietary fiber, ethanol or industrial methylated spirits (IMS)

are added and the insoluble and precipitatable soluble dietary fiber is captured, washed with ethanol and acetone, dried and weighed. One of the duplicate residues is analyzed for protein, and the other for ash. Non-precipitable dietary fiber in the filtrate is recovered by concentrating, then desalting through ion exchange resins, concentrating and quantitating by LC.

B. Apparatus

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

(b) *Digestion Bottles.*— 250 mL Duran^{R} glass bottles with plastic caps or 250 mL polypropylene bottles with polypropylene caps.

(c) *Fritted crucible.*— Büchner, fritted disk, Pyrex® 60mL, pore size, coarse, ASTM 40-60 µm, Corning No. 36060® or equivalent. Prepare as follows:

i. *Ash* overnight at 525°C in muffle furnace, cool furnace to 130°C before removing crucibles to minimize breakage.

ii. Remove any residual Celite and ash material by using a vacuum

iii. Soak in 2 % cleaning solution, *[C(i)]* at room temperature for 1 hr.

iv. Rinse crucibles with water and deionized water.

v. For final rinse, use 15 mL acetone and air dry.

vi. Add approximately 1.0 g Celite to dried crucibles and dry at 130°C to constant weight.

vii. Cool crucible in desiccators for approximately 1 hr and record mass of crucible containing Celite.

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

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

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

(g) *Water bath(s).*— rotary motion or horizontal shaking, large-capacity (20-24 L) with covers; capable of maintaining temperature of 37+/-1°C and 60+/-1°C; equipped with automatic timers for on-off

operation or equivalent (e.g. Grant® OLS 200 shaking incubation bath). Assure that shaking action /sample agitation in water bath used is sufficient to maintain sample solids in suspension, and no residue build up or rings of sample material form in the digestion bottle during the enzymatic digestions, for example, a back and forth shaker with the bottles placed at 45° will provide adequate agitation while placing the bottle vertically or horizontally will not.

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

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

(j) *Timer.*

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

(l) *pH meter.*

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

(n) *Dispensers.*—

i. 15 ± 0.5 mL for 78 % EtOH (or IMS), 95 % ethanol (or IMS), and acetone.

ii. 40 ± 0.5 mL for buffer.

(o) *Cylinder-*,Graduated, 500 mL

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

(q) *Rubber spatulas*

(r) *Muffle furnace*.— 525 ± 5°C

(s) *Glass or polypropylene columns.*—, 20 cm x 2.5 cm id; to hold ion exchange resins with fittings and plastic tubing for filling and draining.

(t) *Liquid Chromatograph (LC).*— With oven to maintain a column temperature of 90 °C and a 50 µL injection loop. Column operating conditions are: Temperature, 90°C; mobile phase, distilled water plus ethylene diamine disodium calcium salt ($Na_2CaEDTA$) (50 mg/L), flow rate, 0.5 mL/min for Sugar-Pak column. System must separate maltose from higher malto-oligosaccharides. . Run time of 30 minutes to assure column cleaned out. Use only distilled water if size exclusion columns are used. System must separate maltose from maltotriose (Figure 3 (a) and (b) and Figure 4 (a) and (b)). Run time of 60 minutes to assure columns cleaned out.

(u) *Guard column (or pre-column).*— Waters Guard Pak LC pre-column inserts (Waters part no. WAT015209) or equivalent.

(v) *LC columns.— Option 1*-Waters Sugar-Pak® 6.5 x 300 mm (part no. WAT085188) or equivalent *Options 2-size exclusion LC Columns*— Two TSK-Gel 30cm x 7.8mm connected in series, G2500PWXL, (Sigma Adrich part no. 808020).

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

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

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

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

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

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

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

(dd) *Rotary evaporator*.—Heidolph Laborota® 4000 or equivalent.

(ee) *Thermometer* –Capable of measuring to 110 C.

C. Reagents

(a) Ethanol 95 % v/v. or *Industrial Methylated Spirits* (IMS). Industrial Methylated Spirits made up of: ethanol 84.83 (w%), 85.95 (v%); water 5.66 (w%), 4.52 (v%); 2-propanol 4.91 (w%), 5.00 (v%); methanol 4.60 (w%), 4.52 (v%). Can be prepared by mixing 5 volumes of 2 propanol with 95 volumes of denatured ethanol formula SDA-3A[100 volumes of 95% ethanol combined with 5 volumes of methanol].

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

(c) Acetone, reagent grade.

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

*(e) *Pancreatic α-amylase (50 Units/mL)/AMG (3.4 Units/mL).* – Immediately before use, dissolve 0.10 gram of purified porcine pancreatic α-amylase (150,000 Units/g; Ceralpha method; AOAC Method

2002.01) in 290 mL of sodium maleate buffer (50 mM, pH 6.0 plus 2 mM $CaCl_2$ and 0.02% sodium azide) and stir for 5 min. Add 0.3 mL of AMG.

(f) **Protease (50 mg/mL; ~ 350 Tyrosine Units/mL) in 50 % v/v glycerol.* – Solution is viscous; for dispensing use positive displacement dispenser. Use as supplied.

(g) *Deionized water*.

(h) *Celite.*— acid-washed, pre-ashed (Megazyme G-CEL100 or G-CEL500).

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

(j) *Sodium maleate buffer.*— 50 mM, pH 6.0 plus 2 mM $CaCl_2$ and 0.02 % sodium azide. Dissolve 11.6 g of maleic acid in 1600 mL of distilled water and adjust the pH to 6.0 with 4 M (160 g/L) NaOH solution. Add 0.6 g of calcium chloride ($CaCl_2.2H_2O$) and 0.4 g of sodium azide [**NOTE:** do not add the sodium azide until the pH has been adjusted. Acidification of sodium azide releases a poisonous gas. Handle sodium azide and maleic acid with caution only after reviewing MSDS, using appropriate personal protective gear and laboratory hood] and adjust the volume to 2 L. Stable for > 1 year at 4°C.

(k) *Trizma* Base *(Sigma cat. no. T-1503), 0.75 M.*— Add 90.8 g of Trizma base to approx. 800 mL of distilled water and dissolve. Adjust volume to 1 L. Stable for > 1 year at room temperature.

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

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

(n) *LC retention time standard.*— Standard source having the distribution of oligosaccharides (DP $\geq$3) corn syrup solids (DE 25; Matsutani Chemical Industry Co., Ltd., Itami City, Hyogo, Japan; www.matsutani.com), analyzed by LC. Dissolve 2.0 g portion of oligosaccharide mixture and 0.50 g maltose in deionized water and transfer to 100 mL volumetric flask. Pipette 10 ml of internal standard *(C(p))*. Bring to volume with 0.02% sodium azide solution *(C(t))*. Transfer solutions to 100 mL polypropylene bottle. Stable at room temperature for one year. When using size exclusion chromatography, sorbitol is replaced by the same mass of glycerol if sorbitol elutes at the same point as glucose on these columns. Alternatively, diethylene glycol can be used with both systems (not employed inb the current study).

(o) *Mixed-bed ion exchange resins for each test portion.— (1) m-1.* — 25 g Amberlite FPA53 (OH^-) resin (Rohm and Haas, France S.A.S.), ion exchange capacity (R-OH exchange capacity data

supplied by manufacturer) 1.6 meq/mL (min) or equivalent and *(2) m-2.*— 25 g Ambersep 200 (H^+) resin or equivalent, (Rohm and Haas, France S.A.S.) are mixed together just prior to use and packed in column *[B(s)]* for analysis of each test portion. The converted resin should satisfy the following specifications: Ion exchange capacity (R-H exchange capacity data supplied by manufacturer) 1.6 meq/mL (min), pH: 4-7. Before mixing and packing the 2 resins into a column, wash each resin with H_2O to obtain a pH value of 7-8 for *m-1* and 4-7 for *m-2*. Note: If using Amberlite 200C "Na-type" resin convert into the H^+ form by mixing 500 mL of resin with 2 L of 1 M HCl in a 5 L beaker. Swirl the suspension occasionally over a 1 h period and then allow the resin to settle and decant supernatant solution. Add 4 L of distilled or deionized water, swirl over 5 min, allow the resin to settle, and then decant supernatant solution. Pour the resin onto nylon filter cloth on a strainer and wash the resin several times with distilled or deionized water until the pH is 4-7. The Amberlite FPA53 resin as purchased has been converted to the OH^- form before shipping and is ready to use. If alternative resins are used, determine that carbohydrates are not retained by the resin by preparing a test solution consisting of 1mL of 100 mg/mL internal standard and 2.5 mL of 10 mg/mL fructooligosaccharides diluted to 10 mL. Proceed to step *H(b)* Recovery of the internal standards and fructooligosaccharides should match that of the solution injected directly onto the LC.

(p) D-Sorbitol.(Stock internal standards for SugarPak system)— 100 mg/mL. Dry analytical grade high purity (>99.5%) D-sorbitol in a freeze-drier at 60°C over 1 day. Weigh 100.00 g D-sorbitol into a beaker, dissolve in water transfer to a 1 L volumetric flask with water, and dilute to volume. Transfer to a polypropylene bottle and add 0.2 g of sodium azide as a preservative [**NOTE:** Handle sodium azide with caution only after reviewing MSDS, using appropriate personal protective gear and laboratory hood}. Seal well. Stable at room temperature for > 1 year. If using the TSK size exclusion chromatographic system, replace D-sorbitol with diethylene glycol.

(q) D-Sorbitol (Working internal standard).—10 mg/mL. Pipette 100 mL of solution *(C(p))* to 1 L volumetric flask and dilute to volume with deionized water. Add 0.2 g sodium azide as a preservative [**NOTE:** Handle sodium azide with caution only after reviewing MSDS, using appropriate personal protective gear and laboratory hood]. Stable at room temperature for > 1 year. If using the TSK size exclusion chromatographic system, replace D-sorbitol with diethylene glycol.

(r) D-Glucose LC standards-(5, 10, 20 mg/mL). Accurately weigh 0.5, 1.0, and 2.0 g portions of high purity (> 99.5 %) D-Glucose -(Sigma Chemical Company) and transfer to 3 separate 100 mL volumetric flasks respectively. To each flask pipette 10 ml of internal standard *(C(q))*. Bring to

volume with 0.02% sodium azide solution *(C(t))*. Transfer solutions to 100 mL polypropylene bottles. Stable at room temperature for one year.

(s) Deionized water containing $Na_2CaEDTA$ (50 mg/L).

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

*Items (d), (e) and (f) are supplied in the Integrated Total Dietary Fibre Assay Kit (K-DFRSOL)) available from Megazyme International Ireland Ltd., Bray Business Park, Southern Cross Road, Bray, County Wicklow, Ireland, but preparations of reagents and buffers which meet the criteria as specified in the method above may also be used.

D. Preparation of Test Samples

Collect and prepare samples as intended to be eaten, i.e. baking mixes should be prepared and baked, pasta should be cooked etc. Defat per AOAC 985.29 if >10% fat. For high moisture samples (>25%) it may be desirable to freeze dry. Grind ca 50 g in a grinding mill ***B(a)*** to pass a 0.5 mm sieve. Transfer all material to a wide mouthed plastic jar, seal, and mix well by shaking and inversion. Store in the presence of a desiccant.

E. Enzyme Purity

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

F. Enzymatic Digestion of Sample

(1) Blanks

With each assay, run two blanks along with samples to measure any contribution from reagents to residue.

(2) Samples

(a) *Weigh*-duplicate 1.000±0.005 g samples accurately into Duran^{R} glass bottles.

(b) *Addition of Enzymes*- Wet the sample with 1.0 mL of ethanol (or IMS) and add 40 mL of pancreatic α-amylase/AMG mixture ***[C(e)]*** to each bottle. Cap the bottles. Transfer the bottles to a Grant OLS 200 shaking incubation bath (or similar) and secure the bottles in place with the springs in the shaker frame.

(c) *Incubation with pancreatic α-amylase/AMG.*— Incubate the reaction solutions at 150 revolutions/min (orbital motion) or the relevant rate to assure suspension in a reciprocal shaker at 37°C for exactly 16 h (e.g. 5.00 pm to 9.00 am).

(d) *Adjustment of pH to approx. 8.2 (pH 7.9-8.4), Inactivation of α-amylase and AMG.*— After 16 h, remove all sample bottles from the shaking water bath and immediately add 3.0 mL of 0.75 M Trizma base solution to terminate the reaction. (At the same time, if only one shaker bath is available, increase the temperature of the shaking incubation bath to 60°C in readiness for the protease incubation step). Slightly loosen the caps of the sample bottles and place the bottles in a water bath (non-shaking) at 95-100°C, and incubate for 20 min with occasional shaking (by hand). Using a thermometer, ensure that the final temperature of the bottle contents is > 90°C (checking of just one bottle is adequate).

(e) *Cool*- Remove all sample bottles from the hot water bath (use appropriate gloves) and cool to approx. 60°C.

(f) Protease treatment.—Add 0.1 mL of protease solution ***[C(f)]*** with a positive displacement dispenser (solution is viscous). Incubate at 60°C for 30 min.

(g) *pH adjustment.*— Add 4.0 mL of 2 M acetic acid to each bottle and mix. This gives a final pH of approx 4.3

(h) Proceed to step G(a) for determination of High Molecular Weight Dietary Fiber.

G. Determination of High Molecular Weight Dietary Fiber

(a) Precipitation of high molecular weight soluble dietary fiber (HMWSDF).— To each sample, add 10 ml of 10 mg/mL internal standard solution C(q), then add 227.5 mL (measured at room temperature) of 95 % (v/v) EtOH (or IMS) *[C(a)]* preheated to 60°C and mix thoroughly. Allow the precipitate to form at room temperature for 60 min.

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

(c) Filtration.— Using vacuum, filter precipitated enzyme digest G(a) through crucible. Using a wash bottle with 78 % (v/v) EtOH or IMS, *[C(b)]* quantitatively transfer all remaining particles to crucible. Retain filtrate and washings (steps c and d) for determination of low molecular weight soluble fiber (step *[H(a)]*).

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

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

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

(g) Protein and ash determination.— The residue from one crucible is analyzed for protein, and the second residue of the duplicate is analyzed for ash. Perform protein analysis on residue using Kjeldahl or combustion methods (Caution should be exercised when using a combustion analyzer for protein in the residue. Celite volatilized from the sample can clog the transfer lines of the unit). Use 6.25 factor for all cases to calculate g of protein. For ash analysis, incinerate the second residue for 5 hr at 525°C. Cool in desiccator and weigh to nearest 0.1 mg. Subtract crucible and Celite weight to determine ash.

(h) Proceed to step H(a).

H. Determination of Low Molecular Weight Soluble Dietary Fiber (LMWSDF)

Note-Proper deionization is an essential part of obtaining quality chromatographic data on LMWSDF. To obtain familiarity regarding the appearance of salt peaks in the LMWSDF chromatograms, dissolve 10 mg of sodium chloride into 10 mL of 10 mg/mL LC internal standard *(C(q))* and proceed to step *H(c)* at "Transfer …….to a 10 mL disposable……". To assure the resins being used are of adequate deionizing capacity, dissolve 50 mg of sodium chloride in 10 mL of deionized water. Add 10 mL of 10 mg/mL LC internal standard *(C(q))*, and proceed to step *H(b)* at "Quantitatively transfer…. to a column…….". The LC chromatogram of this solution should show no peaks in the time range corresponding to carbohydrates of DP3 or greater.

(a) *Filtrate recovery, deionization, and LC analysis.*— (Set aside the filtrate from one of the sample duplicates *G(d)* to use in case of spills or if duplicate LMWDF data is desired. Transfer approximately one half of filtrate *G(d)* of the other sample duplicate to a 1-L evaporator flask and concentrate with a rotary evaporator to near dryness at 50°C. Repeat with remaining half of filtrate.

(b) *Deionization of sample.*— Dissolve the residue in approx. 20 mL of deionized water, and transfer quantitatively to a sealable container if planning to store overnight before deionization. Quantitatively transfer to a column (25 cm x 2.5 cm id), *[C(o)]* containing 25 g each, thoroughly mixed, Amberlite FPA 53 (OH^-) (*C(o)m-1*) and Ambersep 200 (H^+) (*C(o)m-2*) prepared just before use, collecting the eluate (eluting at a consistent rate of 0.5 to 2 mL per min) into a 500 mL round bottom rotary evaporator flask. Continue to elute extract through the column with 200 mL deionized water at a consistent rate of 0.5 to 2.0 mL/min. Evaporate to near dryness at 50°C.

(c) *Preparation of samples for LC and LC analyses.*— Quantitatively transfer the concentrate to a 10 mL volumetric flask (using a few mL of deionized water to rinse the flask) and dilute to volume (10 mL) with deionized water. Transfer the contents of the 10 mL volumetric flask to a 10 mL disposable syringe *(B(bb))*, and filter through a 0.45 µm filter*(B(y))* **.** Use a 100 µL LC glass syringe *(B(cc))*, to fill the 50 µL injection loop on the LC *(B(t).* Perform this analysis in duplicate.

(d) *Determine the response factor for D-glucose;* (Since D-glucose provides an LC RI response equivalent to the response factor for the non digestible oligosaccharides that make up low molecular weight soluble dietary fiber, the LC is calibrated using D-glucose, and the response factor is used for

determining the mass of LMWSDF). Use a 100 µL LC syringe *(B(cc))*, to fill the 50 µL injection loop for each standard internal standard/D-glucose solution *(C(r))*. Inject in triplicate.

–Internal Standard Method: Obtain the values for the peak areas of D-glucose and internal standard from the 3 chromatograms. The reciprocal of the slope obtained by comparing the ratio of peak area of D-glucose/peak area of internal standard (y-axis) to the ratio of the mass of D-glucose/mass of internal standard (x-axis) is the "response factor". Determine the average response factor (typically 0.91 for D-sorbitol, or 0.73 for diethylene glycol).

Response factor (Rf) = (PA-IS) / (PA-Glu) x (Wt-Glu / Wt-IS)

where:

PA-Glu = peak area D-glucose; PA-IS = peak area internal standard;

Wt-Glu = mass of D-glucose in standard; Wt-IS = mass of internal standard in standard.

–External Standard Method Obtain the values for the peak areas of D-glucose from the 3 chromatograms. Determine the average response factor:

Response factor (Rf) = (Wt-Glu) / (PA-Glu)

where:

PA-Glu = peak area D-glucose;

Wt-Glu = mass of D-glucose in standard;

(e) *Calibrate the area of chromatogram to be measured for LMWSDF:* Use a 100 µL LC syringe *(B(cc))*, to fill the 50 µL injection loop with retention time standard *(C(n))*. Inject in duplicate. Determine

demarcation point between DP 2 and DP 3 oligosaccharides (disaccharides sucrose and maltose versus higher oligosaccharides). See figures 3 (a) and (b) and 4 (a) and (b).

(f) *Determine peak area of LMWSDF(PA LWMSDF) and internal standard (PA internal standard) in chromatograms of sample extracts*-Inject sample extracts *(H(c))* on LC. Record area of all peaks of DP greater than the DP2/DP3 demarcation point as PA LMWSDF. Record the peak area of internal standard as PA-IS.

I. Calculations for HMWDF

Blank (B, mg) determination:

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

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

HMWDF :

HMWDF (mg / 100g) = [(R1 + R2)/2 –P – A – B] / (M1 + M2) / 2] x 100

where:

R1 = residue mass 1 from M1 in mg; R2 = residue mass 2 from M2 in mg;

M1 = test portion mass 1 in g; M2 = test portion mass 2 in g;

A = ash mass from R1; P = protein mass from R2

J. Calculations for low molecular weight soluble dietary fiber (LMWSDF)

-Internal Standard Method

LMWSDF (mg/100g) = Rf x (Wt IS, mg) x (PA LMWSDF)/(PA IS) x 100/M.

Where:

Wt-IS is mg of internal standard contained in 10 mL of internal standard solution pipetted into sample filtrate.
PA LMWSDF is the peak area of the low molecular weight dietary fiber
PA IS is the peak area of the internal standard.
M is the test portion mass M1 or M2 of the sample whose filtrate was concentrated and analyzed by LC.

-External Standard Method

LMWSDF (mg/100g) = Rf x (PA LMWSDF) x 100/M.

Where:

PA LMWSDF is the peak area of the low molecular weight dietary fiber
M is the test portion mass M1 or M2 of the sample whose filtrate was concentrated and analyzed by LC.

K. Calculation of Total Dietary Fiber

Total Dietary Fiber (%) = (HMWDF + LMWSDF)/1000

Precollaborative Ruggedness Testing

A precollaborative ruggedness study was conducted with a number of laboratories to assure adequate method performance. A call was placed for volunteer laboratories to participate in evaluating the methodology. Volunteer laboratories were sent 6 samples along with copies of the method, ion exchange resins, and a supply of enzymes. Each laboratory was requested to run each sample in singlet. Laboratories were requested to conduct the analysis, ask questions regarding procedures and write-up, and provide feedback to the study directors on any aspects of the method for which the collaborator might have a concern. The results of the analysis on the ruggedness testing samples are shown in Table 4. Relevant comments received from the participating laboratories were incorporated as changes to improve the method

as appropriate. No procedural changes were found to be necessary, and only minor edits of the text for clarity were put in place.

As can be seen, the results of the precollaborative ruggedness study were typical for dietary fiber methods. Repeatability, reproducibility, and the HORRAT were within the range of performance characteristics typically found for dietary fiber methods wherein a significant number of manual steps are necessary to carry out the assay. The between laboratory variability s_R ranged from 0.53 to 1.57 for total dietary fiber, and the between laboratory relative variability RSD_R ranged from 3.12 to 12.66% . This is due to the fact that all dietary fiber methods to date are comprised of a significant number of technique dependent manual operations, each of which contributes to the overall variability of the final results. By way of comparison, the statistical characteristics of the various AOAC Official Methods of analysis are compiled in Table 3. The statistical characteristics of this method, which combines steps from AOAC Official Methods 985.29, 991.43, 2001.03, and 2002.02, lie within the ranges of the statistical characteristics of the current Official Methods.

Therefore, the study directors determined the method was ready for full collaborative study.

Collaborative Study Protocol

Eight food samples were selected for the collaborative study. In as much as the method under consideration incorporates resistant starch and nondigestible oligosaccharides into a more traditional dietary fiber methodology, the samples for this collaborative study were chosen to be challenging, i.e. with emphasis on quantitating products high in resistant starch (legumes, resistant starch ingredient, and whole grain products) and products with typical levels of nondigestible oligosaccharides (all samples). Methods designed to quantitate "more traditional dietary fiber" have been thoroughly studied and validated since 1980 (e.g. AOAC 985.29, 991.43, etc). Inclusion of components such as resistant starch and NDO in the CODEX Alimentarius definition indicates that updated testing procedures must include the capability of accurately quantitating these components.

Moist samples were freeze dried before grinding. All samples were ground to method specific size and homogenized by thorough mixing before being subdivided into polyethylene bottles and sealed. Samples,

copies of the method, report sheets, and sample storage instructions, along with an adequate supply of enzymes and deionizing resins were shipped to collaborating laboratories by express overnight shipment. A total of 18 laboratories reported data for the collaborative study samples. One laboratory reported little or no LMWSDF for their assays. Subsequent investigation revealed an ion exchange resin has undergone a color change, however the reason for loss of LMWSDF not fully determined. Another laboratory utilized High Pressure Anion Exchange Chromatography with electrochemical detection instead of the prescribed LC method. Data from these two laboratories are not included in data tables or statistical analysis.

Statistical Treatment

Collaborating laboratory data were evaluated statistically according to AOAC protocols using AOAC-supplied software. Of the 128 valid pairs of assay results reported, laboratories 1, 2, 3, 4, 5, 8, 9, 11, 13, and 16 had no statistical outliers, laboratories 6, 12, 14, and 15 had one statistical outlier, laboratory 7 had 3 statistical outliers, and laboratory 10 had 4 statistical outliers, for a total of 11 statistical outliers overall. The data from laboratories 17 and 18 were not included in the statistical evaluation due to operational issues as reported by those laboratories. The paired data from the blind duplicate results reported by the collaborating laboratories are shown in Table 1. Outliers, and their reason for removal , are indicated and foot noted in Table 1.

Results and Discussion

To simulate food digestion in the small intestine, a combination of gentle shaking combined with enzymatic digestion at 37°C is used. Temporary heating to 100°C destroys the amylase and amyloglucosidase activity and promotes some denaturation of protein, providing for efficient protein digestion after cooling to 60°C. Incorporating the high molecular weight dietary fiber segregation steps and the low molecular weight dietary fiber quantitation from previously adopted Official Methods of Analysis completes the assay.

The raw data results of the dietary fiber collaborative study are shown in Table 1. Cochran and Grubb's outliers are noted directly in the table. Table 2 shows the statistical results obtained after removal of outliers as described in the statistics section above. As stated above, samples for this collaborative study were chosen to be challenging, i.e. with emphasis on quantitating products high in resistant starch (legumes, resistant starch ingredient, and whole grain products) and products with typical levels of nondigestible oligosaccharides (all samples). As can be seen, the within laboratory variability s_r ranged from 0.41 to 1.43, and the between laboratory variability s_R ranged from 1.18 to 5.44. Again, as with the precollab results,

when compared to the dietary fiber statistical results in Table 3, this level and range of variability are similar to those of other dietary fiber methods, influenced by the significant number of technique dependent manual operations, each of which contributes to the overall variability of the final results.

COLLABORATORS' COMMENTS

One collaborator comment was that LMWSDF results were slightly higher when calculated using the external standard method than when using the internal standard method with sorbitol as the internal standard. The collaborator believes this is due, in part, to the difficulty of properly integrating the peak area of the sorbitol in so far as it is not always baseline resolved on both sides. The study directors understand this problem and are seeking an alternate internal standard that does not demonstrate these deficiencies, however they do not believe this had a significant impact on the results of this study.

Another collaborator comment concerned the use of Duran bottles rather than beakers for the enzymatic digestion steps of the method. The cap on the Duran bottles retains liquid that must be carefully recovered during filtering. It is also more difficult to transfer traces of dietary fiber from the neck of the bottle to the filter than it is with a beaker as used in AOAC 985.29 and 991.43. Duran bottles were chosen, in conjunction with the shaker, to assure that the entire sample being tested properly contacts the enzymes during digestion. Use of the bottle and shaker does not allow rings of sample to form above the solution and keeps the mix homogeneous during the entire digestion.

RECOMMENDATION

The study directors recommend this method be adopted as an Official First Action Method of AOAC International.

ACKNOWLEDGEMENTS

The study directors wish to acknowledge and thank the collaborators for all their efforts in completing this study.

TABLE 1: Collaborative Study Data for Total Dietary Fiber (%TDF reported by laboratories)

SAMPLE/ LAB #	Bran Cereal		Broccoli, Freeze Dried		Carrots, Freeze Dried		Haricot Beans		Resistant Starch		Red Kidney Beans		Wh
1	31.60	31.54	30.38	30.25	25.94	24.94	50.34	50.40	45.86	46.10	24.87	24.05	12.
2	30.64	31.25	30.44	30.14	24.89	25.64	48.26	46.80	47.16	47.76	24.10	25.06	11.
3	30.78	34.47	33.74	32.60	26.66	25.87	49.73	50.02	45.09	42.02	27.30	27.25	11.
4	29.79	29.23	28.31	28.11	23.08	23.09	33.07	34.89	48.63	49.96	24.17	24.82	11.
5	29.10	29.80	27.60	28.20	23.60	23.80	48.90	49.60	42.90	42.10	22.20	21.70	10.
6	32.46	34.38	32.12	33.57	25.81	26.83	53.29	53.79	54.28	52.49	25.57	26.28	14.
7	36.61	34.62	29.66	30.55	24.28	23.74	54.90	52.49	49.34	50.57	32.16[C]	24.10[C]	33.4
8	30.83	30.04	29.34	29.08	26.10	26.20	42.39	40.11	44.76	40.80	23.51	23.76	12.
9	28.23	29.11	29.52	30.09	24.11	24.16	47.56	48.72	45.11	44.71	23.44	23.29	12.
10	33.61 C	24.51 C	27.82	26.22	20.27 DG	18.97 DG	41.06	42.76	34.06	34.46	22.71[C]	17.21[C]	12.
11	31.97	32.29	30.93	30.91	25.98	26.15	50.14	49.85	45.54	45.84	25.78	25.52	14.
12	29.65	28.65	30.11	32.52	*	20.22 DG	48.20	46.97	36.31	40.50	21.31	20.95	5.
13	32.33	31.69	32.75	30.35	25.68	25.00	56.40	51.93	44.80	44.72	29.24	28.31	11.
14	29.22	29.35	28.95	28.92	24.03	23.41	38.04 C	48.80 C	35.87	35.81	21.73	22.17	10.
15	32.69	31.61	31.98	31.83	27.74 C	25.15 C	44.48	47.84	43.24	44.44	24.73	23.91	13.
16	31.60	31.78	33.07	33.30	25.80	25.82	50.37	49.50	42.74	42.79	23.70	24.00	10.

*** Laboratory reported no result for this sample.**
C. Cochran test outlier on total dietary fiber
SG Single Grubb's test outlier on total dietary fiber
DG Double Grubb's test outlier on total dietary fiber

TABLE 2 Statistics on Total Dietary Fiber

Sample/ Parameter	Bran Cereal	Broccoli, Freeze Dried	Carrots, Freeze Dried	Haricot Beans	Resistant Starch	Red Kidney Beans	Whole Grain Bread	Whole Grain Pasta
# of Labs	15	16	13	15	16	14	15	13
Mean %	31.24	30.42	25.02	47.83	44.09	24.38	11.57	12.65
S_r	0.94	0.78	0.41	1.36	1.28	0.42	1.43	0.57
S_R	2.00	1.95	1.18	5.44	5.05	2.10	2.08	1.43
RSD_r	3.01	2.55	1.65	2.83	2.89	1.71	12.34	4.47
RSD_R	6.42	6.42	4.70	11.38	11.45	8.6	17.97	11.31
HORRAT	2.69	2.68	1.91	5.09	5.06	3.48	6.49	4.14

Table 3. Comparable AOAC method Data

METHOD NUMBER	TITLE	S_r	RSD_r	S_R	RSD_R	HORRAT
985.29	Total Dietary Fiber in Foods	0.15 – 0.99	0.56 - 66.25	0.27 – 1.36	1.58 – 66.25	0.76 – 17.46
991.42	Insoluble Dietary Fiber in Food and Food Products	0.41 – 2.82	0.86 – 10.38	0.62 – 9.49	3.68 – 19.44	1.73 - 8.68
991.43 *	Insoluble Dietary Fiber in Food and Food Products	0.36 – 1.06	1.50 – 6.62	0.85 – 2.06	1.58 – 12.17	0.74 – 4.66
992.16	Total Dietary Fiber	0.18 – 1.01	1.48 – 14.73	0.22 – 2.06	4.13 – 17.94	1.84 – 4.62
993.19	Soluble Dietary Fiber in Food and Food Products	0.49 - 1.15	1.74 – 5.93	0.79 – 2.05	2.41 – 7.01	1.13 – 2.83
994.13	Total Dietary Fiber (Determined as Neutral Sugar Residues, Uronic Acid Residues, and Klason Lignin)	0.32 – 2.88	1.80 – 6.96	0.52 – 4.90	4.80 – 11.30	2.32 – 4.20
2001.03	Dietary Fiber Containing Supplemented Resistant Maltodextrin (RMD)	0.02 - 1.63	1.33 – 6.10	0.04 – 2.37	1.79 – 9.39	0.77 – 3.32
2002.02	Resistant Starch in Starch and Plant Materials	0.08 – 2.66	1.97 - 4.12	0.21 – 3.87	4.58 - 10.9	1.44 – 3.74

*Samples that were not dried and/or desugared only

Table 4: Results of Precollaborative Ruggedness Testing (% TDF reported by laboratories).

Lab #/SAMPLE	Whole Grain Bread	Defatted Peanuts	Haricot Beans	Resistant Starch	Defatted Soybean	Low Carbohydrate Spaghetti
1	6.23 [a]	14.13	51.05	43.19	23.51	4.64
2	10.58	12.51	50.69	41.24	21.82	6.75
3	11.45	11.45	50.25	42.70	23.70	7.70 [a]
4	9.75	15.21	45.46[a]	41.50	21.03	4.70
5	11.13	16.43[a]	51.27	41.35	24.06	5.92
6	10.05	14.16	49.33	41.69	21.43	5.18
7	10.25	18.76[a]	51.23	46.20[a]	21.16	9.70 [a]
8	9.86	14.67	47.85	41.56	20.59	4.80
9	9.97	12.37	47.63	41.53	18.96[a]	4.38
10	13.94[a]	13.22	52.42	44.64	22.19	5.25
11	10.53	13.73	51.01	44.01	23.13	5.43
12	10.33	14.25	50.34	38.61[a]	22.42	5.61
13	8.43[a]	23.33[a]	48.19	39.99	23.29	4.72
14	9.80	14.00	52.50	43.40	22.90	5.10
15	8.85[a]	12.36	49.27	43.21	15.41[a]	3.04[a]
AVERAGE	10.33	13.51	50.22	42.31	22.40	5.21
S_R	0.53	1.12	1.57	1.32	1.13	0.66
RSDR%	5.11	8.30	3.12	3.12	5.04	12.66

a. Statistical Outlier

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Figures:

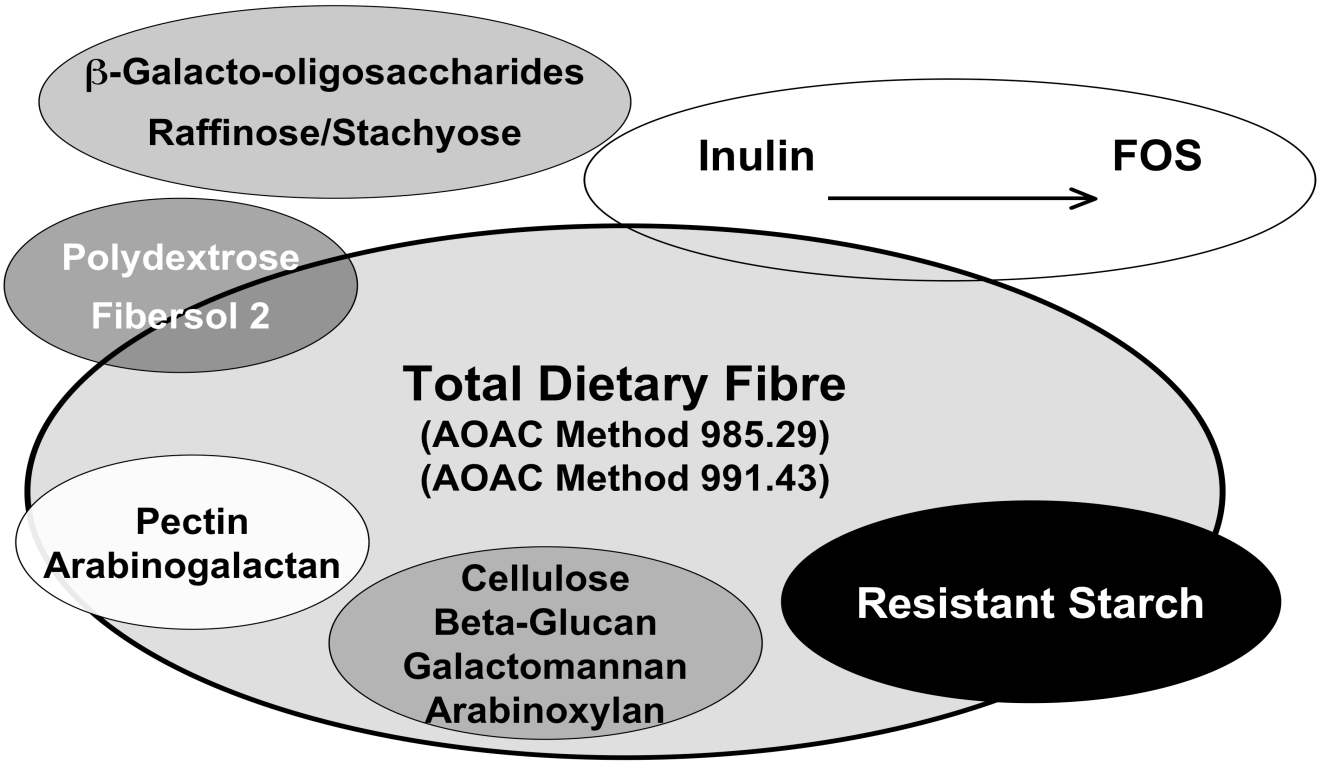

Figure 1. Schematic representation of dietary fiber components measured, and not measured, by AOAC Official Methods 985.29 and 991.43. Also depicted are the problems of partial measurement of RS, Polydextrose and resistant maltodextrins by current AOAC total dietary fiber methods. Most of the LMWSDF (galactooligosaccharides, fructooligosaccharides etc) are not measured. The current integrated total dietary fiber procedure measures all components shown, with no possibility of double counting.

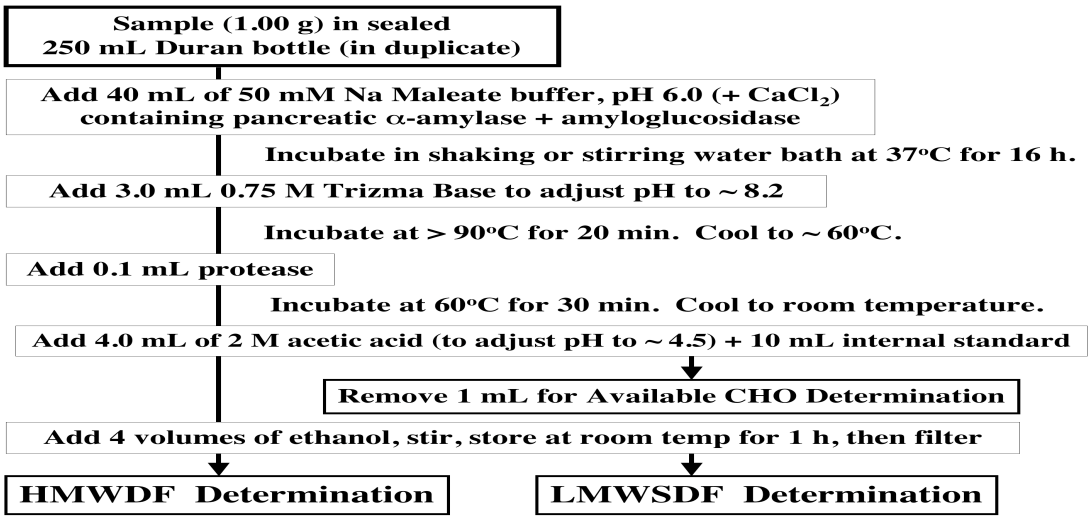

Figure 2. Schematic representation of the integrated total dietary fibre procedure, also showing where samples can be removed for determination of available carbohydrates (not part of this study).

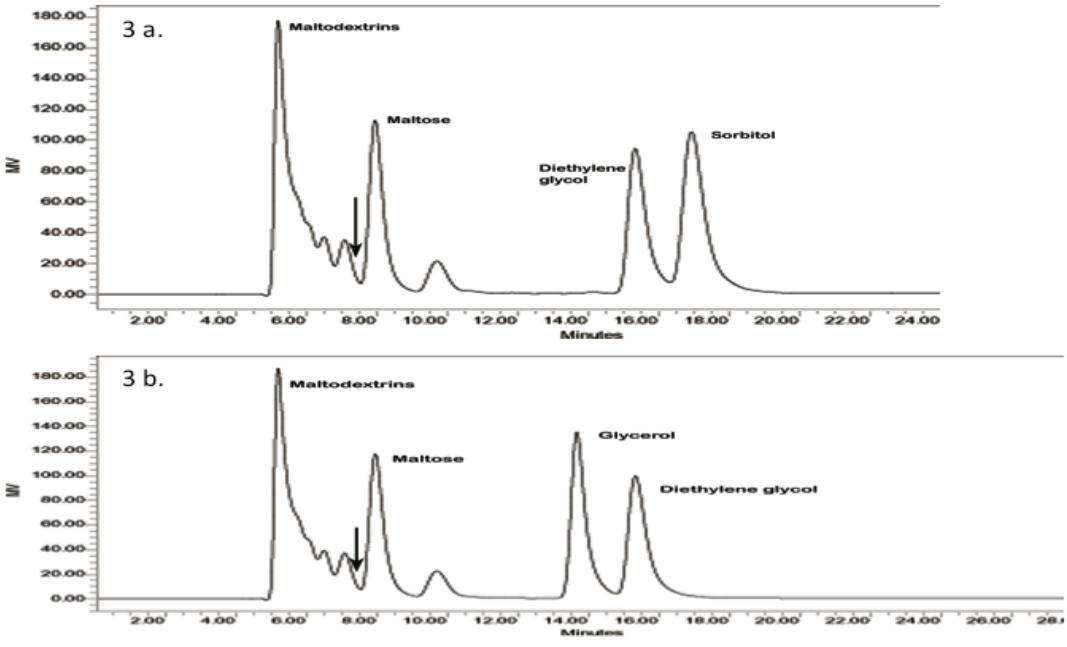

Figure 3. Chromatography of a mixture of maltodextrins, maltose, diethylene glycol plus either (a) D-sorbitol, or (b) glycerol on a Waters Sugar Pak column. The arrows show demarcation between DP 2 (maltose) and DP 3 (higher maltodextrins).

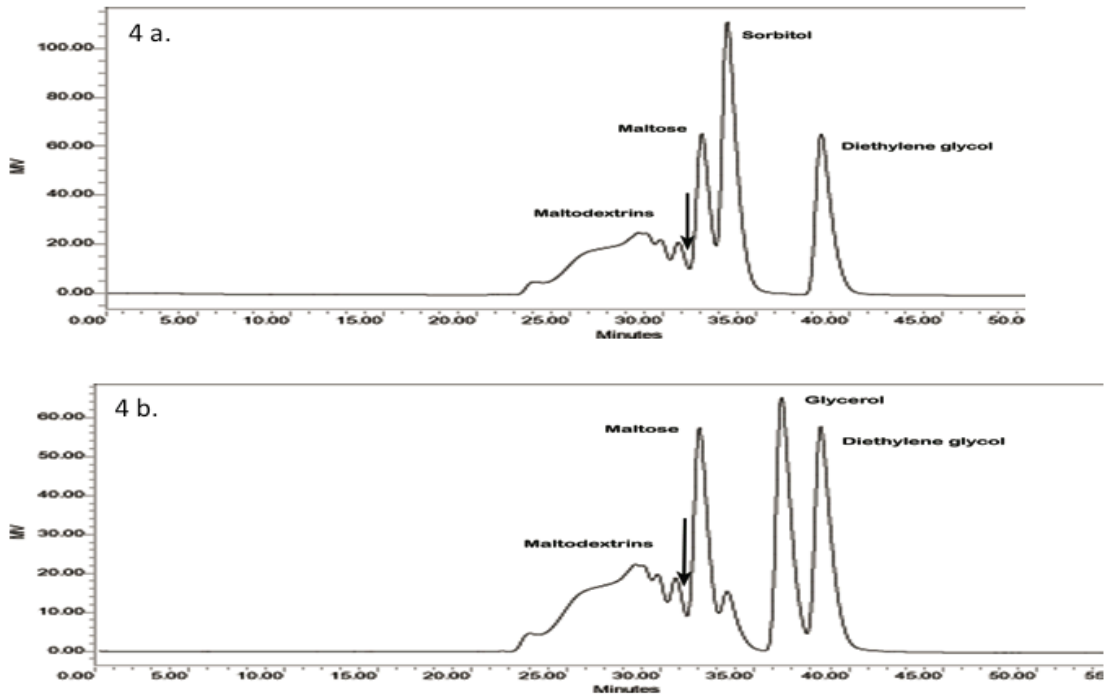

Figure 4. Chromatography of a mixture of maltodextrins, maltose, diethylene glycol plus either (a) D-sorbitol, or (b) glycerol on two TSK gel filtration columns (G2500PWXL) in series. The arrows show demarcation between DP 2 (maltose) and DP 3 (higher maltodextrins).