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ORIGINAL PAPER

An integrated procedure for the measurement of total dietary fibre (including resistant starch), non-digestible oligosaccharides and available carbohydrates

Barry V. McCleary

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Abstract A method is described for the measurement of dietary fibre, including resistant starch (RS), non-digestible oligosaccharides (NDO) and available carbohydrates. Basically, the sample is incubated with pancreatic α-amylase and amyloglucosidase under conditions very similar to those described in AOAC Official Method 2002.02 (RS). Reaction is terminated and high molecular weight resistant polysaccharides are precipitated from solution with alcohol and recovered by filtration. Recovery of RS (for most RS sources) is in line with published data from ileostomy studies. The aqueous ethanol extract is concentrated, desalted and analysed for NDO by high-performance liquid chromatography by a method similar to that described by Okuma (AOAC Method 2001.03), except that for logistical reasons, D-sorbitol is used as the internal standard in place of glycerol. Available carbohydrates, defined as D-glucose, D-fructose, sucrose, the D-glucose component of lactose, maltodextrins and non-resistant starch, are measured as D-glucose plus D-fructose in the sample after hydrolysis of oligosaccharides with a mixture of sucrase/maltase plus β-galactosidase.

Keywords Total dietary fibre · Resistant starch · Non-digestible oligosaccharides · Available carbohydrates · Integrated method

B. V. McCleary (✉)
Megazyme International Ireland Limited,
Bray Business Park, Southern Cross Road,
Bray, County Wicklow, Ireland
e-mail: barry@megazyme.com

Introduction

> "Clearly, a physiological basis for the definition of dietary fiber is necessary. If it were not for the physiological effects of dietary fiber, there would be no interest in the subject on the part of researchers, consumers, regulators, and manufacturers. The objective of ethical nutrition research is to determine those physiological factors (nutrition factors) that improve and optimize the quality of life in terms of increasing life span, and/or improved health resulting from improved body function and increased overall comfort. The term dietary fiber was coined and its definition refined based on observations of positive health effects related to consumption of diets rich in this component" [1].

> "The definition and analysis of dietary fibre are intimately related. Analysis methods have to be developed in accordance with the conceptual definitions, but in practice, compromises must be accepted due to constraints of cost and time. All types of dietary components can be separated at different levels of complexity and determined separately for research purposes, though short-hand methods are needed for labelling and control purposes" [2].

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

The term “dietary fibre” was coined by Hipsley [3] to cover the non-digestible constituents of plants that make up the plant cell wall, known to include cellulose, hemicellulose and lignin. The aim was to define some property of the constituent of the food that could be related to physiological behaviour in the human small intestine. In 1976, Trowell et al. [4] broadened the dietary fibre definition to become primarily a physiological definition, based on edibility and resistance to digestion in the human small intestine. Thus, the definition included indigestible polysaccharides such as gums, modified celluloses, mucilages and pectin, and non-digestible oligosaccharides (NDO).

In developing a method to meet this analytical requirement, effort focussed on the removal of starch and protein. It was essential that the enzymes employed were sufficiently active, as well as being devoid of contaminating activities acting on dietary fibre components. Following extensive international collaboration, the method that evolved was AOAC Official Method 985.29 “Total dietary fiber in foods; enzymatic-gravimetric method” [5, 6]. Subsequently, the method was extended to allow measurement of total dietary fibre (TDF), soluble dietary fibre and insoluble dietary fibre (IDF) in foods (AOAC Official Method 991.43) [7]. Various other modified methods for fibre have been approved by AOAC International [8].

In 1998, the American Association of Cereal Chemists began a critical review of the current state of dietary fibre science, including consideration of the state of the dietary fibre definition. Over the course of the following year, the committee held three workshops and provided an international Web site, available to all Web users worldwide, to receive comments. All interested parties were provided with additional opportunity for comment. After due deliberation, an updated definition of dietary fibre was delivered to the AACC Board of Directors for adoption in early 2000, and published [9], 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.”

In parallel research in the UK, methods were developed by Englyst et al. [10–13] for the measurement of non-starch polysaccharides (NSP), based on the original work of Southgate et al. [14, 15]. This NSP procedure measures only NSP; resistant starch (RS) and NDO are excluded. Starch in the sample is completely dissolved in hot dimethyl sulphoxide (DMSO), diluted in buffer and depolymerised with thermostable α-amylase followed by a mixture of pancreatin and pullulanase. The NSP recovered is acid-hydrolysed to monosaccharides which are measured by high-performance liquid chromatography (HPLC), gas–liquid chromatography (after derivatisation) or colorimetrically.

Several definitions of dietary fibre have appeared over the past 5 years. The Food Nutrition Board (FNB) of the Institute of Medicine of the National Academies (USA) (2001) [16] defined dietary fibre 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 definition of dietary fibre that arose from the 27th session of the Codex Committee on Nutrition and Foods for Special Dietary [17] was similar in many respects to that proposed by AACC, 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 an FAO/WHO Expert Consultation on Carbohydrates in Human Nutrition held in Geneva on 17–18 July 2006 [18] (as discussed at the 28th session of the Codex Committee on Nutrition and Food for Special Dietary Uses [19], the participants concluded that the definition of dietary fibre should be more closely linked to fruits, vegetables and wholegrain cereals. To achieve this aim, they stated that the definition should include (1) a source element identifying that the dietary fibre is an intrinsic component of these food groups and (2) a chemical element identifying the component to be measured. The following definition was proposed: “Dietary fibre consists of intrinsic cell wall polysaccharides”. In rationalising this decision and definition, many points were discussed, including their statement that NSP can be measured specifically and that NSP relate directly to the content of plant cell walls, and the other beneficial substances they contain such as micronutrients and phytochemicals.

The focus of the FAO/WHO proposed definition [18] is based on the measurement of plant cell walls, rather than on

carbohydrates that are not digested or absorbed in the human small intestine. Consequently, this does not address the sole reason for which there is an interest in dietary fibre, i.e. “the belief that dietary fibre contributes positively to the health/quality of life of the consumer”. The current obesity epidemic, with associated type 2 diabetes, highlights the need to incorporate more non-digestible or slowly digested carbohydrate into the human diet. Clearly, this objective has not been achieved to date even though the health benefits of eating more fruits, vegetables and cereals have been advocated for decades. There is an urgent and essential need to incorporate more non-digestible polysaccharides and NDO in the types of foods (usually processed) that consumers choose to eat. To reject out of hand all of the work undertaken over the past 10 years on non-digestible carbohydrates such as RS, β-glucan, galactomannan, psyllium gum and a range of NDO such as fructo-oligosaccharides (FOS), resistant maltodextrins (RMD) and galacto-oligosaccharides (GOS) cannot be justified, and will have a major negative effect on efforts to include more dietary fibre in foods that consumers choose to eat. In appendix 2 of the FAO/WHO report [18], numerous arguments for an NSP-based definition and against the AACC International/AOAC International definition and methods for dietary fibre are given. It was concluded that NSP methodology gives an accurate and reliable measure of these components. Ignored are the facts that (1) in the removal of starch with hot DMSO, there is partial depolymerisation of pectin, resulting in subsequent loss on alcohol precipitation, (2) during acid hydrolysis a percentage of the monosaccharides are converted to furfural and methylfurfural (at varying rates) and lost analytically, and (3) no colorimetric method gives the same colour response with different monosaccharides, and thus a colorimetric method cannot be used to give a true quantitative measure of NSP in varying matrices because the ratio of the monosaccharides is not known.

The fact that the AOAC procedures for measurement of dietary fibre (e.g. AOAC Method 985.29 and AOAC Method 991.43) do not quantitatively measure RS and, in general, measure little of the NDO, is well known to researchers and analysts in the field. Methods have thus been developed for measurement of specific NDO, namely AOAC Method 997.08 [20] and AOAC Method 999.03 [21] for FOS, AOAC Method 2000.11 [22] for Polydextrose®, AOAC Method 2001.03 [23] for Fibersol 2®, and AOAC Method 2001.02 [24] for GOS, and for the accurate measurement of RS, i.e. AOAC Method 2002.02 [25]. The need for an integrated procedure for the measurement of dietary fibre (including RS) and of all of the NDO as a group was discussed in a methods group meeting at the conference “Dietary Fibre 2006” [26]. Various approaches aimed at resolving this analytical challenge were proposed, and the method currently described is one of these approaches.

Experimental

Materials

Chemicals and enzymes

D/L-Maleic acid (cat. no. M-0375), bovine serum albumin (BSA; cat. no. A-2153), DMSO (cat. no. D-8779) and sodium azide (cat. no. S-8032) were from Sigma-Aldrich Ireland, Dublin, Ireland . Acetic acid (glacial) general reagent, sodium hydroxide and calcium chloride ($CaCl_2 \cdot 2H_2O$) were from Merck, Darmstadt, Germany. Amyloglucosidase (AMG; cat. no. E-AMGDF), thermostable α-amylase (cat. no. E-BLAAM), protease (cat. no. E-BSPRT), sucrase (cat. no. E-SUCR), β-galactosidase (cat. no. E-BGLAN) and crude panceatic α-amylase were obtained from Megazyme International Ireland, Bray, Ireland.

Polysaccharides

Barley β-glucan (medium viscosity; cat. no. P-BGBM), citrus pectin (cat. no. P-CITPN) and wheat arabinoxylan (cat. no. P-WAXM) were from Megazyme. Partially degraded chicory inulin (Raftilose P-95®) was a kind gift from Raffinerie Tirlemontoise, Tienen, Belgium. Polydextrose® (Litesse®) was from Danisco, New York, USA and Fibersol 2® was from Matsutani Chemical Company, Hyogo, Japan. Regular maize starch (lot 60401; RMS) and high-amylose maize starch (HAMS; lot 60107) were 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, USA. Native potato starch was from Avebe, Foxhol, The Netherlands. ActiStar® (enzyme-modified tapioca/cassava starch; US Patent 6,043,229) was from Cerestar, Vilvoorde, Belgium. Potato amylose (cat. no. A-9262) and ACS soluble starch (cat. no. S-9765) were from Sigma Chemical Company.

Assay kits

D-Fructose/D-glucose assay kit (cat. no. K-FRUGL), α-amylase assay kit (Ceralpha; cat. no. K-CERA), total starch assay kit (cat. no. K-TSTA), TDF assay kit (cat. no. K-TSTA), D-sorbitol/xylose assay kit (cat. no. K-SORB) and RS assay kit (cat. no. K-RSTAR) were obtained from Megazyme International Ireland, Bray, Ireland.

Methods

Measurement of TDF (including RS) in cereal, vegetable, fruit and food products

Introduction This method is modelled on AOAC Method 2002.02 [25, 27] for measurement of RS. The enzymes used are essentially devoid of activity on dietary fibre components and NDO. The AMG and pancreatic α-amylase enzyme preparations are devoid of activity on pectin and FOS and have negligible activity on β-glucan. Activity on RMD is consistent with reported information on partial hydrolysis of the oligosaccharides by α-amylase and AMG [22, 23]. The protease employed is devoid of α-amylase (an essential requirement in this assay format). This procedure measures IDF, high molecular weight soluble dietary fibre (HMWSDF), RS and high molecular weight NDO.

Principle Samples (1 g) in 250-mL Duran® bottles are incubated in a shaking incubation bath at 150 rpm in orbital mode, with pancreatic α-amylase and AMG for exactly 16 h at 37 °C. During this time, non-resistant starch is solubilised and hydrolysed to D-glucose by the combined action of the two enzymes. The pH is adjusted to approximately 8 and the reaction solutions are incubated at 100 °C to inactivate α-amylase and AMG and to denature protein. Denatured protein is digested at 60 °C with protease. The pH is adjusted to approximately 4.5 with acetic acid. [If a sample (0.5 mL) is to be removed for available carbohydrates (ACH) determination, it is done at this point.] Four volumes of ethanol is added with mixing, to precipitate soluble, polymeric dietary fibre (including RS that is solubilised, but not depolymerised, in the 100 °C incubation step) and to remove depolymerised protein and D-glucose (derived from depolymerised, non-resistant starch). The suspension is filtered. The residue is washed with 76% ethanol, 95% ethanol, and acetone; dried, and weighed. One duplicate is analysed for protein and the other is incubated at 525 °C to determine ash. The TDF is the weight of the filtered and dried residue less the weight of the protein and ash. The ethanolic wash solution can be concentrated, desalted and analysed by HPLC to determine the NDO content (see "Measurement of low molecular weight NDO"). If ACH is to be measured, a 0.5-mL aliquot is removed from the reaction solution after the point where the pH is adjusted with acetic acid (see "Measurement of ACH").

Apparatus

1. *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.
2. *Duran® bottles*: 250 mL with caps.
3. *Fritted crucible*: Corning no. 36060 Büchner, fritted disk, Pyrex® 60 mL, pore size, coarse, ASTM 40–60 μm, or equivalent. Prepare as follows:
   - (a) Ash overnight at 525 °C in a muffle furnace.
   - (b) Remove Celite and ash material by using a vacuum.
   - (c) Soak in 2% Micro cleaning solution at room temperature for 1 h.
   - (d) Rinse crucibles with water and deionised water.
   - (e) For the final rinse, use 15 mL acetone and air-dry.
   - (f) Add approximately 1.0 g Celite to dried crucibles and dry at 130 °C to constant weight.
   - (g) Cool the crucible in a desiccator for approximately 1 h and record the weight of the crucible containing Celite.
4. *Filtering flask*: Heavy-walled, with 1-L side arm.
5. *Rubber ring adaptors*: For use on filtering flasks.
6. *Vacuum source*: Vacuum pump or aspirator with a regulator capable of regulating the vacuum.
7. *Water bath*: Rotary motion, shaking, large capacity (20–24 L) with covers; capable of maintaining temperatures of 37 and 60 °C; equipped with automatic timers for on–off operation, e.g. Grant OLS 200 shaking incubation bath.
8. *Balance*: 0.1-mg accuracy.
9. *Ovens*: Two, mechanical convection, set at 103±2 and 130±3°C.
10. *Timer.*
11. *Desiccator*: Airtight, with SiO_2 or equivalent desiccant. Desiccant dried biweekly overnight in a 130 °C oven.
12. *pH meter.*
13. *Pipettors and tips*: 50–200-μL and 5-mL capacity.
14. *Dispensers*
    - (a) 15±0.5 mL for 78% ethanol, 95% ethanol and acetone.
    - (b) 40±0.5 mL for buffer.
    - (c) Cylinder, 500 mL.
15. *Magnetic stirrers and stirring bars.*
16. *Rubber spatulas.*
17. *Muffle furnace*: 525±5 °C.

Reagents

1. *Ethanol*, 95% v/v.
2. *Ethanol*, 76%: Place 207 mL water into a 1-L volumetric flask. Dilute to volume with 95% ethanol. Mix.
3. *Acetone*, reagent grade.
4. *Enzymes for TDF/RS assay* (Megazyme International Ireland). Store at 0–5 °C

(a) *AMG solution, 5 mL, 3,300 U/mL in 50% v/v glycerol*. Solution is viscous; for dispensing use a positive displacement dispenser. AMG solution is stable for up to 5 years when stored at 4 °C. (Note: One unit of enzyme activity is the amount of enzyme required to release 1 μmol glucose from soluble starch per minute at 40 °C and pH 4.5.) AMG solution should be free of detectable levels of free glucose.
(b) *Pancreatic α-amylase (40 U/mL) plus AMG (3 U/mL)*. Immediately before use, suspend 0.30 g pancreatic α-amylase (30,000 U/g; Ceralpha method) in 220 mL sodium maleate buffer (50 mM, pH 6.0 plus 2 mM $CaCl_2$ and 0.02% sodium azide) and stir for 5 min. Add 0.2 mL AMG.
(c) *Protease (E-BSPRT); 50 mg/mL; 350 tyrosine units/mL*. Use as supplied.

5. *Deionised water*.
6. *Celite*: Acid-washed, preashed (Megazyme G-CEL100 or G-CEL500).
7. *Cleaning solution*: Micro (International Products, Trenton, NJ, USA). Make a 2% solution with deionised water.
8. *Sodium maleate buffer*: 50 mM, pH 6.0 plus 2 mM $CaCl_2$ and 0.02% sodium azide. Dissolve 11.6 g maleic acid in 1,600 mL distilled water and adjust the pH to 6.0 with 4 M (160 g/L) NaOH solution. Add 0.6 g calcium chloride ($CaCl_2{\cdot}2H_2O$) and 0.4 g sodium azide and adjust the volume to 2 L. The buffer is stable for more than 1 year at 4 °C. Note: Do not add the sodium azide until the pH has been adjusted. Acidification of sodium azide releases a poisonous gas.
9. *Trizma base (Sigma cat. no. T-1503), 0.75 M*: Add 90.8 g Trizma base to approximately 800 mL distilled water and dissolve. Adjust the volume to 1 L. The solution is stable for more than 1 year at room temperature.
10. *Acetic acid solution, 2 M*: Add 115 mL glacial acetic acid (Fluka 45731) to a 1-L volumetric flask. Dilute to 1 L with distilled water.
11. *pH standards*: Buffer solutions at pH 4.0, 7.0 and 10.0.

Measurement of IDF and HMWSDF

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

Samples

1. Weigh duplicate 1.000±0.005 g samples accurately into 250-mL Duran® bottles.
2. Wet the sample with 1.0 mL ethanol and add 40 mL pancreatic α-amylase/AMG mixture in maleate buffer/$CaCl_2$/sodium azide solution (pH 6.0) to each bottle and seal 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.
3. Incubation with pancreatic α-amylase plus AMG. Incubate the reaction solutions at 150 rpm (orbital motion) at 37 °C for exactly 16 h (e.g. 5.00 pm to 9.00 am).
4. Adjustment of pH to approximately 8.2 (pH 7.9–8.4). 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, increase the temperature of the shaking incubation bath to 60 °C ready for the protease incubation step.)
5. Inactivation of α-amylase and AMG. 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 shaking (by hand) a few times during the 20 min. Using a thermometer, ensure that the final temperature of the bottle contents is more than 90 °C (checking of just one bottle is adequate).
6. Cool and protease treatment. Remove all sample bottles from the hot water bath and cool to approximately 60 °C. Add 0.1 mL protease solution with a positive displacement dispenser (solution is viscous) and incubate at 60 °C for 30 min.
7. pH adjustment. Add 4.0 mL of 2 M acetic acid to each bottle and mix. This gives a final pH of approximately 4.3.
   Quantitatively remove 0.50 mL of this solution for ACH determination (if this analysis is to be performed). See "Measurement of ACH" for determination of ACH.
8. Precipitation of HMWSDF. To each sample, add 180 mL of 95% (v/v) ethanol preheated to 60 °C and mix thoroughly. Allow the precipitate to form at room temperature for 60 min.
9. Filtration setup. Tare the crucible containing Celite to the nearest 0.1 mg. Wet and redistribute the bed of Celite in the crucible, using 15 mL of 78% (v/v) ethanol from a wash bottle. Apply suction to the crucible to draw Celite onto fritted glass as an even mat.
10. Filtration. Filter the precipitated enzyme digest (filtrate 1) through the crucible. Using a wash bottle with 78% (v/v) ethanol, quantitatively transfer all remaining particles to the crucible.
11. Wash. Using a vacuum, wash the residue successively with two 15-mL portions of the following:

(a) 76% (v/v) ethanol
(b) 95% (v/v) ethanol
(c) Acetone

Retain this filtrate (filtrate 2) for determination of low molecular weight NDO.

12. Dry the crucible containing the residue overnight in a 103 °C oven.
13. Cool the crucible in desiccators for approximately 1 h. Weigh the crucible containing the dietary fibre residue and Celite to the nearest 0.1 mg. To obtain weight of the residue, subtract the tare weight, i.e. the weight of the dried crucible and Celite.
14. Protein and ash determination. One residue from each type of fibre is analysed for protein, and the second residue of the duplicate is analysed for ash. Perform protein analysis on the residue using the Kjeldahl method. Use a factor of 6.25 for all cases to calculate the number of grams of protein. For ash analysis, incinerate the second residue for 5 h at 525 °C. Cool in a desiccator and weigh to the nearest 0.1 mg. Subtract the crucible and Celite weight to determine weight of ash.

Calculations for IDF and HMWSDF

$$\text{Dietary fibre}(\%) = \frac{\frac{R_1+R_2}{2} - p - A - B}{(m_1+m_2)/2} \times 100,$$

where R_1 is residue weight 1 from m_1, R_2 is residue weight 2 from m_2, m_1 is sample weight 1, m_2 is sample weight 2, A is ash weight from R_1, p is protein weight from R_2 and

$$B = \frac{\mathrm{BR}_1 + \mathrm{BR}_2}{2} - \mathrm{BP} - \mathrm{BA},$$

where BR is blank residue, BP is blank protein from BR_1 and BA is blank ash from BR_2.

Measurement of low molecular weight NDO

Introduction This method is modelled on AOAC Method 2001.03 “Total dietary fibre in foods containing resistant maltodextrins” [23]. In that method, the low molecular weight RMD (LMWRMD) that are soluble in 78% v/v ethanol are recovered and analysed by HPLC. In the current method, the same principle is used to measure all of the low molecular weight NDO (LMWNDO) likely to be in the food product or to have been added. The aqueous ethanol filtrate from the TDF method (filtrate 2) is concentrated by rotary evaporation, desalted by ion-exchange chromatography, concentrated and analysed by HPLC. The differences from AOAC Method 2001.03 are that a Waters Sugar-Pak® 6.5 mm×300 mm (part no. WAT085188) column is used, and D-sorbitol (in place of glycerol) is used as the internal standard.

Principle The aqueous ethanol filtrate recovered from the TDF method [filtrate 2], which contains the LMWNDO, is concentrated by rotary evaporation. The sample is desalted, concentrated and analysed by HPLC. The amount of NDO is determined from the combined area under the curve for all fractions (larger than disaccharide) relative to that for a D-sorbitol internal standard. The relative response factors for D-glucose, D-fructose and D-galactose compared with D-sorbitol are the same.

Apparatus

1. *Equipment as used in the TDF method.*
2. *Duran® glass or plastic columns*: To hold ion-exchange resins, 20 cm×2.5-cm inner diameter; with plastic tubing.
3. *HPLC*: With an oven to maintain a column temperature of 90 °C and a 50-μL injection loop. Column operating conditions are 90 °C; mobile phase, distilled water plus EDTA (50 mg/L), flow rate, 0.5 mL/min.
4. *Guard column (or precolumn)*: Waters Guard Pak HPLC precolumn inserts (Waters part no. WAT015209) or equivalent.
5. *HPLC columns*: Waters Sugar-Pak® 6.5 mm×300 mm (part no. WAT085188) or equivalent.
6. *Detector*: Refractive index; maintained at 50 °C.
7. *Data integrator or computer*: For peak area measurement.
8. *Filters for the disposable syringe*: 0.45-μm membrane, 13 mm.
9. *Filters for water*: 0.45 μm, 47 mm.
10. *Filter apparatus*: To hold a 47-mm, 0.45-μm filter; to filter larger volumes of water.
11. *Syringes*: 10 mL, disposable, plastic.
12. *Syringes*: Hamilton 100 μL, 710SNR syringe.
13. *Rotary evaporator*: Heidolph Laborota 4000 or equivalent.

Reagents

1. *Reagents as used in the TDF method*.
2. *Liquid chromatography retention time standard*: Standard source having the distribution of oligosaccharides (DP 3–5) in the LMWNDO fraction of NDO; corn syrup solids (DE 25; Matsutani Chemical Industry, Itami City, Hyogo, Japan), analysed by liquid chromatography.
3. *Mixed-bed ion exchange resins for each test portion*: (1) 25 g Amberlite FPA53 (OH^-) (Rohm and Haas, France), or equivalent, and (2) 25 g Amberlite 200 C (H^+) (Rohm and Haas, France), or equivalent, are mixed and packed in column for analysis of each test portion. The converted resin should satisfy the following specifications: (1) total ion-exchange capacity 1.74 meq/mL (minimum); (2) effective ion-exchange capacity (R-H exchange capacity) 1.6 meq/mL (minimum); (3) pH 4–7. Before mixing and packing the two

resins into a column, wash each resin with H_2O to obtain a pH value of 7–8 for Amberlite FPA53 (OH^-) and 4–7 for Amberlite 200 C (H^+) . Convert Amberlite 200 C "Na-type" resin into the H^+ form by mixing 500 mL 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 the supernatant solution. Add 4 L distilled or deionised water, swirl over 5 min, allow the resin to settle, and then decant the supernatant solution. Pour the resin onto a nylon filter cloth on a strainer and wash the resin several times with distilled or deionised water until the pH is 4–7.

4. *D-Sorbitol (HPLC standard)*: 10 mg/mL. Dry analytical grade D-sorbitol in a freeze-drier at 60 °C over 1 day. Weigh 10.00 g of dry D-sorbitol of more than 99.5% purity into a small beaker. Quantitatively transfer it to a 1-L volumetric flask with repeated washes with distilled water, and dilute to volume. Add 0.2 g sodium azide as a preservative. Take the purity and weight of D-sorbitol into consideration when calculating the concentration of the final D-sorbitol HPLC standard. Confirm the concentration of D-sorbitol with the D-sorbitol assay kit (cat. no. K-SORB).
5. *D-Sorbitol (for D-glucose-D-sorbitol standard)*: 100 mg/mL. Weigh 10.00 g high-purity D-sorbitol into a small beaker, transfer it to a 100-mL volumetric flask with water, and dilute to volume. Transfer the solution to a 100-mL Duran® bottle and add 0.02 g sodium azide as a preservative. Seal well. The standard is stable at room temperature for more than 1 year.
6. *D-Glucose*, *D-fructose and D-galactose*: HPLC grade, high purity of more than 99.5% from Sigma Chemical Company.
7. *Deionised water containing EDTA (50 mg/L)*.
8. *Sodium azide solution (0.02% w/v)*: Add 0.2 g sodium azide to 1 L deionised water and dissolve by stirring. The solution is stable at room temperature for more than 1 year.

Measurement of LMWNDO

Filtrate recovery, desalting, and LC analysis Add filtrate 2, to a 1-L evaporator flask (in two portions) and concentrate it with a rotary evaporator to near dryness. Dissolve the residue with a minimum amount of deionised water, and transfer the solution quantitatively to a 100-mL Duran® bottle. Add 10 mL of 10 mg/mL D-sorbitol HPLC standard and dilute to volume with deionised water. Transfer the contents of the 100-mL Duran® bottle to a column (25 cm× 2.5-cm inner diameter), *BIV(c)* containing 25 g each, thoroughly mixed, of Amberlite FPA53 (OH^-) and Amberlite 200 C (H^+) prepared just before use. Wash the extract through the column with 200 mL deionised water at a rate of 0.5 mL/min.

Collect the eluate and washings from the ion-exchange column and quantitatively transfer them (in two portions) into a 500-mL round-bottom rotary evaporator flask and evaporate to near dryness. Quantitatively transfer the concentrate to a 10-mL volumetric flask (using a few millilitres of deionised water to rinse the flask) and dilute to volume with deionised water. Transfer the contents of the 10-mL volumetric flask to a 10-mL disposable syringe and filter through a 0.2-μm filter. Use a 100-μL HPLC glass syringe to fill the 50-μL injection loop on the high-performance liquid chromatograph). Perform this analysis in duplicate.

Determine the response factor for D-glucose; D-glucose is equivalent to RMD (LMWRMD; Fibersol 2® and Polydextrose®) and to D-fructose and D-galactose in HPLC response Each chromatogram must be evaluated or standardised for the refractive index response of NDO. This is accomplished using the 10 mg/mL D-sorbitol standard. The peak areas, representing concentration, obtained by HPLC analysis of equal amounts of D-glucose, D-fructose and D-galactose are essentially equivalent. D-Sorbitol is used as the internal standard but its peak area compared with the peak area of an equal amount of D-glucose (or D-fructose, D-galactose, LMWRMD) is not equivalent. A D-sorbitol standard curve is therefore prepared to obtain a "response factor" to calculate the exact amount of LMWRMD, or other LMWNDO such as FOS and GOS, in a chromatogram of each test portion.

Prepare three solutions in individual 100-mL volumetric flasks containing the same amount of D-sorbitol and three levels of D-glucose. It is important to know and use the reported content (i.e. more than 99.5% purity) of both D-sorbitol and D-glucose standards as reported by suppliers. Accurately weigh 0.5, 1.0, and 2.0 g of dry D-glucose into three separate 100-mL volumetric flasks, respectively. To each flask add 10 mL of the 100 mg/mL D-sorbitol standard. Dilute the contents of each flask to volume with a solution of sodium azide (0.02% w/v), and transfer the solutions to 100-mL Duran® bottles and seal the bottles well. These three bottles represent the standard solutions to calculate the "response factor" for D-glucose (D-fructose and D-galactose) that is used to determine the amount of LMWNDO as displayed in liquid chromatography chromatograms. These solutions are stable at room temperature for approximately 1 year.

Use a 100-μL HPLC syringe to fill the 50-μL injection loop for each standard D-sorbitol/D-glucose solution. Obtain the values for the peak areas of D-glucose and D-sorbitol from the three chromatograms. The reciprocal of the slope obtained by comparing the ratio of peak area of D-glucose to

the peak area of D-sorbitol (y-axis) to the ratio of the weight of D-glucose to the weight of D-sorbitol (x-axis) is the "response factor". The average "response factor" is 0.91.

$$\text{Response factor} = 1/(\text{PA}\cdot\text{Glu}/\text{PA}\cdot\text{Sor}) \times (\text{Wt}\cdot\text{Sor}/\text{Wt}\cdot\text{Glu}),$$

where PA·Glu is the peak area of D-glucose, PA·Sor is the peak area of D-sorbitol, Wt·Glu is the weight of D-glucose in the standard and Wt·Sor is the weight of D-sorbitol in the standard.

A flow diagram for a combined enzymatic-gravimetric method and liquid chromatography determination is shown in Fig. 1.

Calculations for LMWNDO All values used in the calculations are in milligrams, except for percentage values.

Calculate the average percentage LMWNDO as follows:

1. LMWNDO (mg/g sample) = [peak area of LMWNDO/peak area of D-sorbitol) D-sorbitol standard mg (in the 10-mL sample volume) × response factor].
2. $\%\text{LMWNDO} = (\text{LMWNDO}^1/\text{SW}^1) \times 100$, where LMWNDO^1 is the weight of LMWNDO^1 from step 1 and SW^1 is the weight of the test portion.
   Repeat the calculations for % LMWNDO^2 using LMWNDO^2 and SW^2.
3. $\%\text{ALMWNDO} = (\%\text{LMWNDO}^1 + \%\text{LMWNDO}^2)/2$, where % ALMWNDO is the average % LMWNDO, % LMWNDO^1 is % LMWNDO^1 for the test portion from step 2 and % LMWNDO^2 is % LMWNDO^2 for the duplicate test portion from step 2 (superscripts 1 and 2 represent HPLC results for duplicate samples).

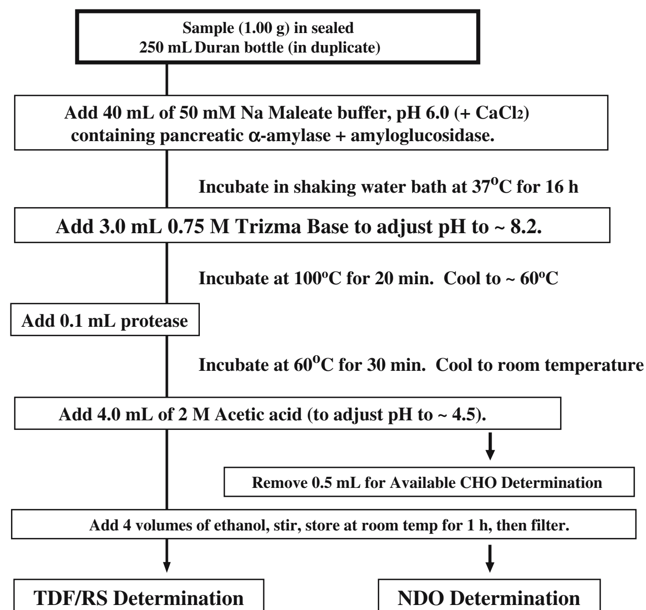

Fig. 1 Schematic representation of the procedure for measurement of total dietary fibre (*TDF*) (including resistant starch, *RS*) showing the points at which samples are removed for non-digestible oligosaccharides (*NDO*) and available carbohydrates (ACH) measurement

Calculate the average percentage TDF as follows:

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

where % (IDF + HMWSDF) is the average percentage of IDF + HMWSDF from "Calculations for IDF and HMWSDF" and % ALMWNDO is the % ALMWNDO from the calculation of the average percentage of LMWNDO above.

Measurement of ACH

Introduction ACH are carbohydrates that are readily hydrolysed into D-glucose and D-fructose and absorbed into the human small intestine. They consist of non-resistant starch, maltodextrins, sucrose, lactose and free D-glucose and D-fructose.

Principle Non-resistant starch and maltodextrins are hydrolysed to D-glucose by the sequential action of thermostable α-amylase and AMG in the TDF procedure (see "Measurement of TDF (including RS) in cereal, vegetable, fruit and food products"). A sample (0.5 mL) of the reaction solution, following pH adjustment with acetic acid, mixing and settling (see "Samples" step 7), is removed and centrifuged in a microfuge, and 0.2 mL is removed and diluted with sodium maleate buffer (pH 6.2). An aliquot of this solution is incubated with a mixture of sucrase/maltase plus β-galactosidase to hydrolyse sucrose and lower DP maltosaccharides (if present in the sample) to D-fructose and D-glucose and lactose to D-glucose and D-galactose. This mixture is analysed for D-glucose and D-fructose using hexokinase plus glucose 6-phosphate dehydrogenase, followed by phosphoglucose isomerase.

Apparatus

1. Spectrophotometer set at 340 nm
2. Disposable plastic cuvettes (1-cm light path, 3.0 mL)
3. Micro-pipettors, e.g. Gilson Pipetman® (20 and 100 µL).
4. Positive displacement pipettor, e.g. Eppendorf Multipette®
   (a) -With 5.0-mL Combitip® (to dispense 0.2-mL aliquots)
   (b) -With 25-mL Combitip® (to dispense 2.0-mL aliquots)
5. Vortex mixer (e.g. IKA® Yellowline Test Tube Shaker TTS2)

Reagents

1. *Sodium maleate buffer (100 mM, pH 6.2) containing BSA (0.5 mg/mL) and sodium azide (0.02% w/v) as a preservative*. Dissolve 11.6 g maleic acid in 800 mL

distilled water and adjust the pH to 6.2 with 4 M (160 g/L) NaOH solution. Add 0.5 g BSA and 0.2 g sodium azide and dissolve. Adjust the volume to 1 L. The solution is stable for more than 1 year at 4 °C.
2. *Imidazole buffer (2 M, pH 7.6) plus magnesium chloride (100 mM) and sodium azide (0.02% w/v) as a preservative*. Use as supplied. The buffer is stable for more than 2 years at 4 °C.
3. *$NADP^+$ (21 mg/mL) plus ATP (42 mg/mL)*. The solution is stable for more than 2 years at −20 °C.
4. *Sucrase (200 U) plus β-galactosidase suspension (8,000 U)*. Freeze-dried powder. Dissolve in 10.5 mL water and store frozen between use. The solution is stable for more than 2 years at 4 °C.
5. *Hexokinase (425 U/mL) plus D-glucose-6-phosphate dehydrogenase (212 U/mL) suspension in 3.2 M ammonium sulphate*. Use as supplied. The solution is stable for more than 2 years at 4 °C.
6. *Phosphoglucose isomerase suspension (1,000 U/mL) in 3.2 M ammonium sulphate*. Use as supplied. The solution is stable for more than 2 years at 4 °C.
7. *D-Glucose plus D-fructose standard solution (0.2 mg/mL of each sugar)*. Use as supplied. The solution is stable for more than 2 years at 4 °C.

Reagents 2–7 are available from Megazyme International Ireland.

Procedure
1. Accurately transfer 0.5 mL of the solution recovered in step 7 of the TDF procedure in “Samples” to a microfuge centrifuge tube and centrifuge at 12,000 rpm for 2 min. Transfer 0.2 mL of the supernatant to a 10-mL volumetric flask and adjust to volume with 100 mM sodium maleate buffer (pH 6.2 (overall dilution of 50-fold) and mix well.
2. Perform all other incubations as described in Fig. 8. Lactose is hydrolysed to D-glucose plus D-galactose by β-galactosidase and sucrose is hydrolysed to D-glucose and D-fructose by the sucrase/maltase enzyme. This enzyme has no action on low DP FOS such as kestose, kestotetraose, kestopentaose or oligosaccharides released on controlled hydrolysis of inulin by *endo*-inulinase (as in Raftilose P-95®).

Calculations Determine the absorbance difference (A_2-A_1) for both the blank and the sample. Subtract the absorbance difference of the blank from the absorbance difference of the sample, thereby obtaining $\Delta A_{\text{D-glucose}}$.

Determine the absorbance difference (A_3-A_2) for both the blank and the sample. Subtract the absorbance difference of the blank from the absorbance difference of the sample, thereby obtaining $\Delta A_{\text{D-fructose}}$.

The concentration of D-glucose and D-fructose (g/100 g) can be calculated as follows:

$$c = \frac{VM}{\varepsilon dv} \times F \times \frac{\text{EV}}{1000} \times \Delta A \times 100,$$

where V is the final volume (millilitres), M is the molecular mass of D-glucose or D-fructose (grams per mole), ε is the extinction coefficient of NADPH at 340 nm (6,300 L/mol/cm), d is the light path (centimetres), v is the sample volume (millilitres), F is the dilution factor (usually 50-fold) and EV is final sample extract volume (45.9±0.05 mL). Empirically, it was found that 1 g of starch, D-glucose or sucrose in aqueous solution occupied a volume of 0.55±0.05 mL. The factor 100 is used to convert the of results to grams per 100 g.

It follows for D-glucose (g/100 g) that

$$c = \frac{2.52 \times 180.16}{6300 \times 1 \times 0.2} \times 50 \times \frac{48.7}{1000} \times \Delta A_{\text{D-glucose}} \times 100$$
$$= 87.74 \times \Delta A_{\text{D-glucose}}.$$

For D-fructose (g/100 g)

$$c = \frac{2.54 \times 180.16}{6300 \times 1 \times 0.2} \times 50 \times \frac{48.7}{1000} \times \Delta A_{\text{D-fructose}} \times 100$$
$$= 88.43 \times \Delta A_{\text{D-fructose}}.$$

For ACH (g/100 g)

$$\text{c} = \text{D} - \text{glucose}(\text{g}/100\text{g}) + \text{D} - \text{fructose}(\text{g}/100\text{g}).$$

Results

While the AOAC methods for the measurement of dietary fibre (AOAC Method 985.29 [5] and AOAC Method 991.43 [7]) have been widely accepted internationally for the measurement of TDF, certain limitations of the methods have been identified over the past 10 years. These problems include underestimation of RS and NDO [28]. In this same period of time, there has been a clear demonstration that these carbohydrate components act physiologically as dietary fibre. With this in mind, several innovative companies have produced RS and various NDO, including FOS, GOS and RMD, for inclusion in processed food products. There is a clear need for a significant increase in the consumption of fibre. Most of these products have been shown to have a positive effect on laxation [29–36], blood glucose attenuation [37–45] and/or blood cholesterol attenuation [46–52].

The problems in measurement of these specific dietary fibre components have led to the development of procedures for measurement of specific dietary fibres: AOAC Method 997.08 and AOAC Method 999.03 for FOS; AOAC Method 2001.02 for GOS; AOAC Method 2000.11 and AOAC Method 2001.03

for RMD; AOAC Method 2002.02 for RS; and AOAC Method 995.16 for β-glucan. While these methods are useful for the measurement of specific dietary fibres, the problem of which method(s) to use arises when an unknown food sample has to be analysed. The problem of measurement of RS has been studied in detail in the author's laboratory [28]. A specific method for RS (Method 2002.02) was developed, but a problem arises when the TDF of a sample is required. AOAC Method 991.43 (TDF) measures all high molecular weight polysaccharides, but it also measures some of the RS. The RS method measures all of the RS, so if the two values are simply added together, there will be an overestimation of dietary fibre by the amount of RS measured by Method 991.43. Various approaches have been investigated to resolve this problem. One of these involved removal of RS from the sample before performing the TDF analysis [28]. This was achieved by preincubating the sample in DMSO before addition of α-amylase. This did result in complete removal of the RS, but the DMSO treatment led to depolymerisation of some polysaccharides such as pectin, with resultant underestimation. An alternative is to dissolve the RS in cold KOH, but on neutralisation and addition of 4 vol of ethanol, a heavy salt precipitate forms, which leads to large ash values and large errors in determination. Another approach investigated was to perform the incubation with α-amylase at a lower temperature (e.g. 80 °C) so that non-resistant starch would still be solubilised and digested, but that RS would be less degraded, and yield values more in-line with RS values obtained with AOAC Method 2002.02 (RS) [53]. For many samples, this did improve the recovery of RS, but for other samples (e.g. native high-amylose starch, banana and ActiStar®) the values were still significantly underestimated.

Since AOAC Method 2002.02 does give a realistic estimate of RS (based on comparisons from ileostomy studies), it was decided to develop a dietary fibre method based on this method. The science behind AOAC Method 2002.02 is not unique. In developing this method, we modelled the format on methods developed by Berry [54], Englyst et al. [55], Champ [56], Goni et al. [57] and Fassant et al. [58]. In analysing various samples, each of these methods gave similar results. However, unlike the other methods, AOAC Method 2002.02 performed well in an interlaboratory evaluation (37 laboratories, 16 samples) [28]. In the current modification of the RS method, sample size had to be increased from 100 mg to 1 g so that there would be sufficient residue to allow accurate gravimetric determination. Incubations were performed in 250-mL Duran® bottles in a shaking incubation bath in orbital motion. This was essential to keep the samples suspended during the incubation, and to yield results in-line with AOAC Method 2002.02. This procedure is outlined in Fig. 1 and the equipment setup is shown in Fig. 2. The sample (1.00 g) is suspended in 40 mL of a solution of pancreatic α-amylase plus AMG in sodium maleate buffer (pH 6.0) containing $CaCl_2$ and sodium azide for 16 h at 37 °C. The reaction is terminated by the addition of Trizma base solution to give a pH of approximately 8.2, and incubation of the bottles in a boiling water bath to raise the temperature to approximately 95 °C. The high pH prevents any further action of AMG on starch as the temperature increases and the RS starts to solubilise. Pancreatic α-amylase still has activity at pH 8.2, but this enzyme is inactivated at approximately 45 °C, a temperature well below the temperature of gelatinisation of the RS (approximately 62–70 °C). This incubation of the sample mixture in a boiling water bath is necessary to ensure denaturation of protein, which in turn is necessary for digestion of the protein by protease. The reaction solution is cooled to 60 °C and protease is added and the incubation is performed for 30 min. At this stage, the pH is adjusted to approximately 4.5 by addition of 2 M acetic acid, and the solution is thoroughly mixed and allowed to settle for approximately 10 min. If ACH are to be determined, a 0.5-mL aliquot of solution is removed and added to an Eppendorf® micro-centrifuge tube for later analysis. This sample is stored in a freezer if it is not analysed on the same day. Four volumes of ethanol is added to the remainder of the solution (approximately 47 mL), and the suspension is stored at room temperature for 60 min. The suspension is filtered and the TDF/RS is determined as

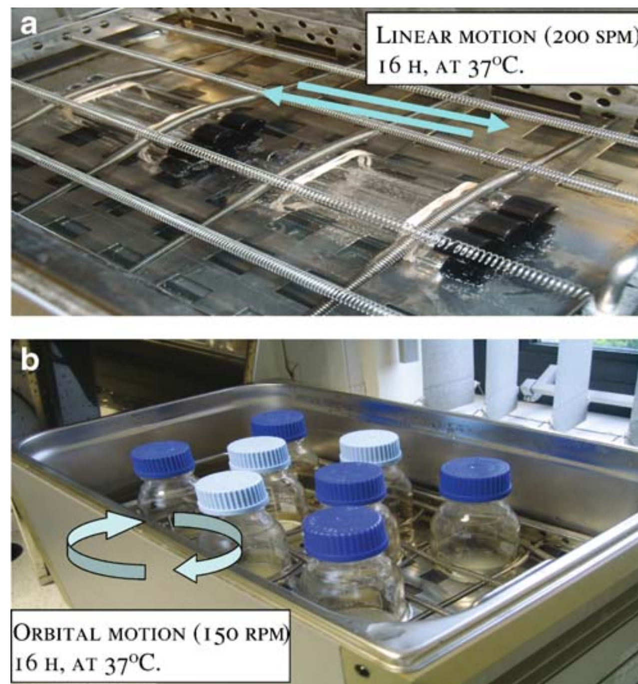

Fig. 2 The equipment and glassware arrangement for incubation of samples with enzymes for measurement of **a** RS (AOAC Method 2002.02) and **b** TDF/RS with the current procedure. In AOAC Method 2002.02, the tubes are shaken at 200 strokes per minute (100 forward and 100 reverse; setting 100 on the bath); in the new TDF/RS procedure, bottles are shaken at 150 rpm

Table 1 Resistant starch (*RS*) values determined for a number of samples using AOAC Method 2002.02 and the current total dietary fibre (*TDF*)/RS procedure

Sample details	RS (% w/w; as-is basis)	
	AOAC Method 2002.02	New TDF/RS method
Native potato starch	64.9	56.8
Actistar®	58.0	48.8
Green banana	51.0	38.0
Hylon VII®	50.0	48.6
Novelose 240®	48.4	44.2
Novelose 330®	38.8	38.7
Hi Maize 1043®	41.0	41.7
CrystaLean®	39.8	37.9
Amylose (potato)	38.2	36.6
Regular maize starch	0.5	0.8
Pinto beans (dry-milled)	39.4	35.6
Haricot beans (dry-milled)	36.9	31.2
Red kidney beans[a]	5.0	5.3
Red Lentils (dry-milled)	7.6	6.1
Flagelot beans (freeze-dried) [a]	5.3	4.5
Cooked/cooled potato	4.0	3.2
Cornflakes	2.2	2.4

Hylon VII® is native high-amylose maize starch. Novelose 240®, Novelose 330®, Hi Maize 1043® and Crystalean® are retrograded high amylose maize starches.

[a.] Samples were freeze-dried with a final moisture content of approximately 2–3%.

described in "Methods". The aqueous ethanol filtrate and washings are retained for determination of LMWNDO.

With use of this new assay procedure, the RS contents of a range of starches, milled grain and some food samples were determined, and the values obtained are shown in Table 1. RS values of the samples were determined by AOAC Method 2002.02 (RS), and by the new TDF/RS procedure but with the modification that RS was determined rather than dietary fibre. The RS was determined using exactly the same procedures as in Method 2002.02, but scaled up by tenfold. Clearly, for all samples except native potato starch, green banana and ActiStar®, the RS values determined by both methods are very similar. Native potato starch, banana starch and ActiStar® completely dissolve in the 100 °C incubation step, and subsequent precipitation by ethanol is apparently not complete. While the RS values for these three samples are underestimated by the current method, it should be noted that with AOAC Method 985.29 or AOAC Method 991.43 the TDF (and thus the RS) value for native potato starch, green banana and ActiStar® is approximately zero. From ileostomy studies, native potato starch has been shown to contain high levels of RS. However, this is a very fragile starch, with the granule structure being readily destroyed by heat or physical treatment; thus, it is unlikely that native potato starch will ever be used as a source of RS in food products. Actistar®, which is prepared by partial hydrolysis of tapioca starch with α-amylase and isoamylase, is also very susceptible to heat treatment. Consequently, ActiStar® can only be used in foods designed for consumption without cooking.

In Table 2, the TDF values are shown for a range of samples traditionally used to check the efficacy and purity of enzymes used in dietary fibre analysis. The values determined for all samples, except for HAMS, are very similar. The much higher dietary fibre value for HAMS reflects a true measure of the RS content of this sample.

The TDF values for a range of RS-containing samples, determined with AOAC Method 991.43 and the new method, are shown in Table 3. In general, the TDF values determined with the new method are much higher than those obtained with AOAC Method 991.43. Just two samples, Novelose 240 and Novelose 330, showed similar values with the two methods. Both samples are retrograded HAMS. Clearly, the new procedure for TDF/RS gives a more accurate measure of dietary fibre in samples containing RS.

The procedure describe in this paper for the measurement of LMWNDO is based on AOAC Method 2001.03 for measurement of LMWRMD. Since in the current procedure the samples are subjected to incubation with pancreatic α-amylase plus AMG for 16 h, followed by heat treatment and incubation with protease, it was important to demonstrate that there is no degradation of the NDO during this process. HPLC traces for Raftilose® (FOS) dissolved in water and analysed compared with the same material subjected to the full enzymic incubation sequence are shown in Fig. 3. The traces are essentially identical, showing that no degradation has occurred. A glycerol peak is evident in the material subjected to enzymic treatment, and this is due to the presence of glycerol as a stabiliser in

Table 2 TDF values determined for a range of samples traditionally used as standards in TDF assays

Sample details	TDF (% w/w; as-is basis)	
	AOAC Method 991.43	New TDF/RS method
β-Glucan	98.0	96.0
Casein	0	0
Pectin	86.5	87
Wheat starch	0.1	0.1
Larch arabinogalactan	83.5	84.0
High-amylose maize starch	29.3	46.5
Wheat arabinoxylan	95.0	94.5

Table 3 TDF values determined for a range of RS-containing samples by AOAC Method 991.43 and the current TDF/RS method

Sample details	TDF (% w/w; as-is basis)	
	AOAC Method 991.43	New TDF/RS method
Hylon VII®	25.6	49.3
Novelose 240®	47.1	44.3
Novelose 330®	35.0	39.9
Actistar®	0.5	47.3
Green banana (freeze-dried)	7.5	37.6
Native potato starch	0.9	64.6
Red kidney beans (freeze-dried)	20.4	21.8
Cooked/cooled potato (freeze-dried)	7.1	9.6
Red lentils	11.3	14.8
Pinto beans (dry-milled)	17.3	54.9
Haricot beans (dry-milled)	23.3	51.9
Regular maize starch	0.1	0.7

All data are reported on an as-is basis. Some of the samples were freeze-dried before analysis. The moisture content of these samples was approximately 2–3%

the AMG and protease enzyme preparations. With Neosugars® (also FOS) similar results were obtained showing no enzymic degradation. With Fibersol 2® (Fig. 4) and Polydextrose® (Fig. 5), it is evident that there is some degradation; however, this is consistent with information supplied by the manufacturers.

The extent of hydrolysis of the various NDO can also be determined by measuring the amount or D-glucose, D-fructose, maltose and sucrose in the various NDO before and after running the samples through the new TDF procedure. The results from such experiments are shown in Table 4. The FOS contain trace levels of D-glucose and significant levels of sucrose; however, these levels do not increase significantly as a consequence of the incubation conditions. The more significant release of D-glucose from Fibersol 2® and Polydextrose® on incubation with α-amylase plus AMG is consistent with information supplied by the manufacturers. The incubation conditions in the new TDF assay cause insignificant degradation of sucrose, as shown by the low level of free D-glucose after incubation. However, incubation of the sample with sucrase enzyme results in quantitative hydrolysis of the sucrose.

In conclusion, the incubation steps in the new TDF procedure cause insignificant degradation of FOS and the degradation of Polydextrose® and Fibersol 2® is in accord with the nature of the oligomers and information provided by the manufacturers. This analysis of the aqueous ethanolic filtrate will give a true measure of the NDO in the original sample.

In AOAC Method 2001.03 (RMD), glycerol is used as the internal standard. However, many enzyme preparations

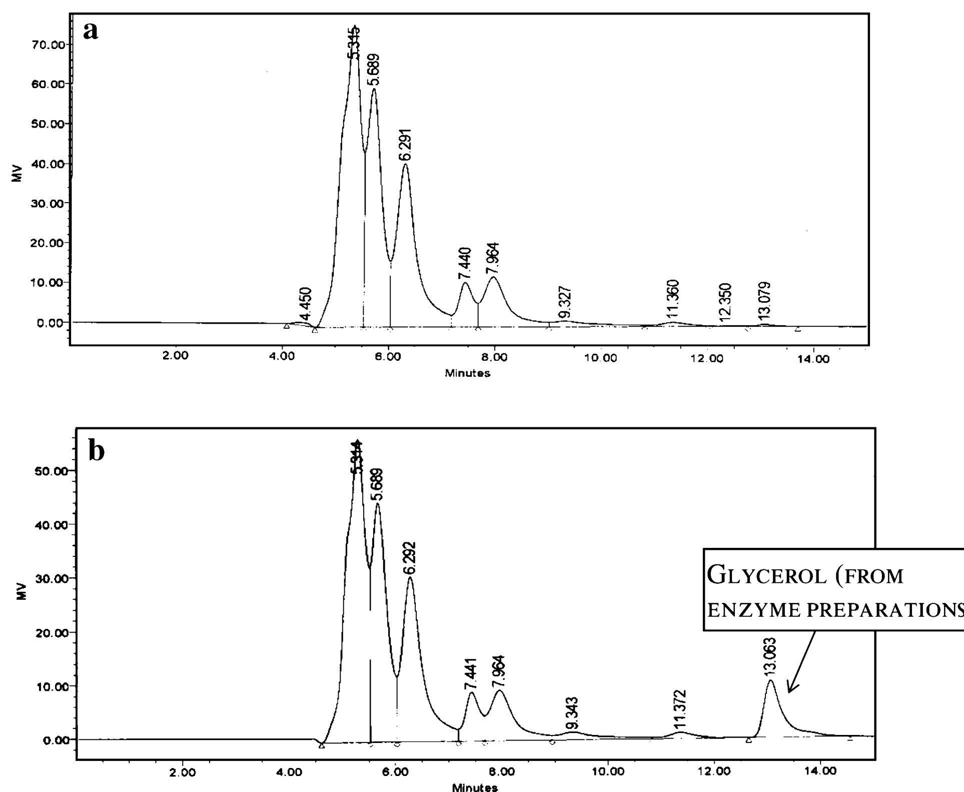

Fig. 3 High-performance liquid chromatography (HPLC) trace for Raftilose® dissolved in water and analysed directly, compared with Raftilose® recovered as NDO after running through the current TDF/RS procedure. Column, Waters Sugar-Pak® (6.5 mm×300 mm). Solvent, distilled water containing EDTA (50 mg/L). Flow rate, 0.5 mL/min. Temperature, 90 °C. **a** No enzyme incubation. **b** Full enzyme incubation

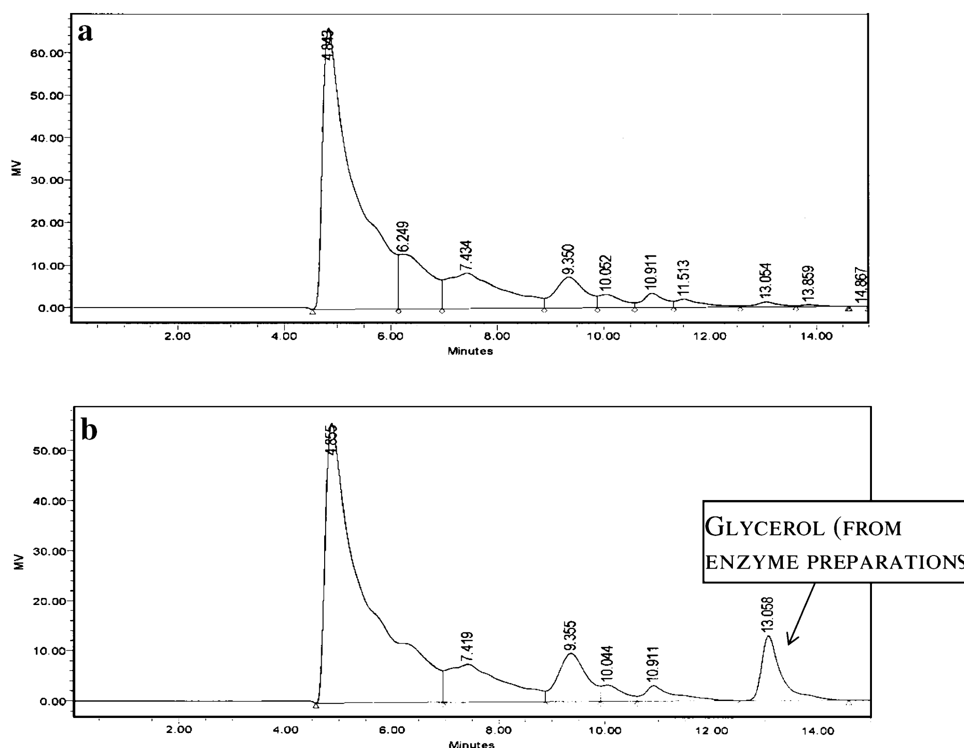

Fig. 4 HPLC trace for Polydextrose® dissolved in water and analysed directly, compared with Polydextrose® recovered as NDO after running through the current TDF/RS procedure. Chromatography conditions as for Fig. 3. **a** No enzyme incubation. **b** Full enzyme incubation

used in TDF assay procedures contain glycerol as a stabiliser. Consequently, a range of other sugars and sugar alcohols were evaluated as a potential replacement for glycerol. Of these, D-sorbitol had the best chromatographic properties (Fig. 6), being eluted from the HPLC column just after glycerol and well away from all of the sugars and oligosaccharides in the food samples.

The HPLC response factors for D-fructose, D-glucose and D-galactose were determined against a D-sorbitol internal standard and the results are shown in Fig. 7. The response

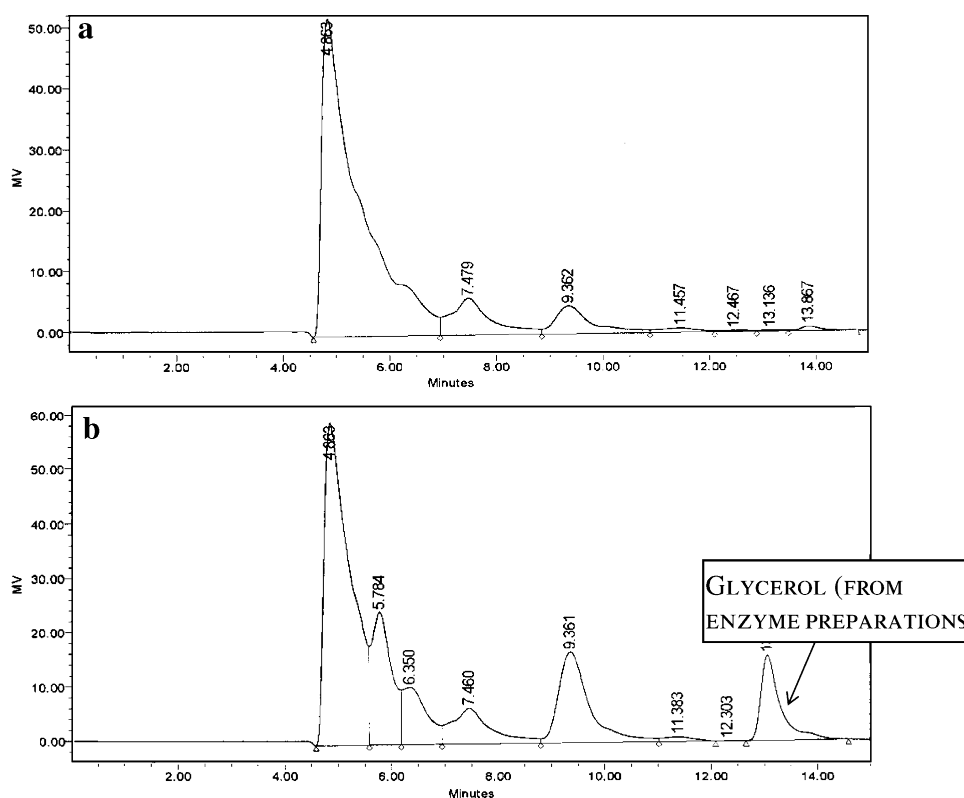

Fig. 5 HPLC trace for Fibersol 2® dissolved in water and analysed directly, compared with Fibersol 2® recovered as NDO after running through the current TDF/RS procedure. Chromatography conditions as for Fig. 3. **a** No enzyme incubation. **b** Full enzyme incubation

Table 4 The extent of hydrolysis of various non-digestible oligosaccharides (measured as released D-glucose and D-fructose) on incubation in the current TDF/RS procedure

Sample	Incubation conditions	Released D-glucose (% w/w)	Released D-glucose + D- fructose + sucrose (% w/w)
Raftilose P-95®	Buffer only	0.04	6.0
	Full enzymic treatment	0.13	6.0
Neosugars® (Litesse®)	Buffer only	0.39	7.5
	Full enzymic treatment	0.59	7.5
Polydextrose®	Buffer only	3.15	4.2
	Full enzymic treatment	4.89	5.7
Fibersol 2®	Buffer only	2.21	3.9
	Full enzymic treatment	9.61	10.1
Sucrose	Buffer only	0.67	99.0
	Full enzymic treatment	0.69	101.3

D-Glucose in the final reaction solution was measured with the hexokinase/glucose 6-phosphate dehydrogenase method (Megazyme cat. no. K-GLUHK). D-Glucose plus D-fructose and sucrose was measured with the sucrose/D-fructose/D-glucose assay kit (Megazyme cat. no. K-SUFRG).

factor for the three sugars is the same; thus, the procedure can be used for measurement of FOS, RMD and GOS, or mixtures of these, i.e. it has universal application for the NDO currently available commercially.

On a physiological basis, edible carbohydrates can be grouped into:

1. Dietary fibre—which includes IDF plus associated lignin, cutin and suberin, HMWSDF and RS.
2. NDO—which are oligosaccharides that are not degraded or absorbed in the human small intestine; they pass into the large intestine, where they are partially or completely fermented. As such, they act physiologically as dietary fibre.
3. Available (glycemic) carbohydrates— those that provide carbohydrate for metabolism. These are digested and absorbed in the small intestine, and include D-glucose, D-fructose, sucrose, maltodextrins, non-resistant starch and the D-glucose component of lactose.

The measurement of dietary fibre and NDO has been discussed. Many methods have been used for the measurement of the various ACH, but from a nutritional point of view, a method that measures these as a group would appear to be useful. In the current procedure, an aliquot of solution is removed from the TDF incubation mixture and analysed for D-glucose and D-fructose, following hydrolysis of sucrose and lactose in the sample. In this procedure, because the aliquot is removed at the end of the 16-h incubation, all of the non-resistant starch in the sample is measured. However, since non-resistant starch can be subdivided into rapidly and slowly digested types, a measure of these two may be desirable. This can be done by removing a sample from the incubation mixture after a predetermined period (probably 1 or 2 h), and analysing this for ACH. The difference between this value and the value obtained for the 16-h sample is "slowly digested starch".

The procedure employed for the measurement of ACH is outlined in Fig. 8, and typical results are shown in Table 5. The procedure gives separate values for D-glucose and D-fructose, which is useful since these sugars have such different glycemic index values [59].

A typical example of measurement of LMWNDO in the aqueous ethanol fraction is shown in Fig. 6. A sample of Kellogg All Bran® was subjected to analysis. The material was analysed according to the TDF/RS and LMWNDO procedure. Sample 1 is All Bran® as purchased; sample 2 is All Bran® to which 100 mg Raftilose P-95® was added and sample 3 is All Bran® to which 200 mg Raftilose P-95® was added. According to the calculations

$$\text{LMWNDO} = \frac{\text{Peak area of LMWNDO}}{\text{Peak area of D} - \text{sorbitol}} \times \text{milligrams of sorbitol} \times \text{response factor.}$$

Thus, for sample 1

$$\text{LMWNDO(mg/g)} = 3.31/12.42 \times 100 \times 0.91 = 24.30;$$

for sample 2

$$\text{LMWNDO(mg/g)} = 15.27/12.01 \times 100 \times 0.91 = 115.8;$$

and for sample 3

$$\text{LMWNDO(mg/g)} = 22.41/19.89 \times 100 \times 0.91 = 206.2$$

The recovery of added Raftilose P-95® was as follows:

- For 95 mg added: 115.18–24.3=91.5 mg
- For 190 mg added: 206.2–24.3=181.9 mg

Note: The amounts of Raftilose P-95® added were 100 and 200 mg. After allowing for the moisture content (approximately 5% w/w), the actual amounts added were 95 and 190 mg.

Fig. 6 HPLC of the low molecular weight NDO (*LMWNDO*) fraction derived from Kellogg All Bran® to which Raftilose P-95® was added at a level of **a** 0 mg, **b** 100 mg (as-is weight) and **c** 200 mg (as-is weight). The glycerol in the sample is derived from the enzyme preparations used; D-sorbitol was added as an internal standard. Chromatographic conditions as for Fig. 3

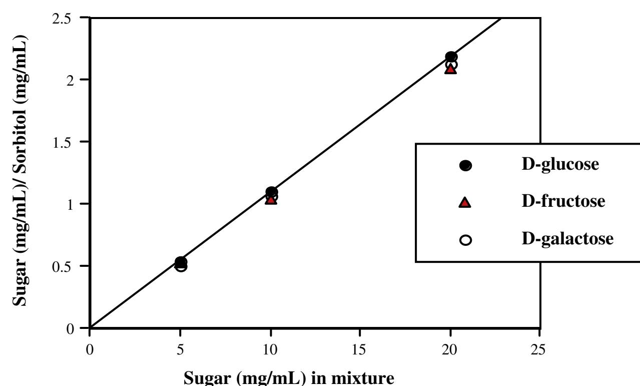

Fig. 7 HPLC response of D-fructose, D-glucose and D-galactose compared with D-sorbitol. Chromatography conditions as for Fig. 3

Discussion

If not for the physiological effects of dietary fibre, there would be no interest in the subject by consumers, regulators, researchers or manufacturers. Because the term "dietary fibre" encompasses a group of components, any method for the measurement of dietary fibre must be closely linked to a definition. In the USA, and most of the rest of the world, the currently accepted definition of dietary fibre is that proposed by AACC International in 2001 [9]. This, in turn, is a refinement of the definition proposed by Trowell [4] in 1976, which was primarily a physiological definition. This definition encompasses the current knowledge on dietary fibre. The outcome of various international surveys in 1992 [60] and 1993 [61] of

Fig. 8 The procedure for the measurement of ACH

Wavelength: 340 nm
Cuvette: 1 cm light path (glass or plastic)
Temperature: 25˚C
Final volume: 2.52 mL (D-glucose)
2.54 mL (D-fructose)
Sample solution: 4-80 mg of D-glucose plus D-fructose per cuvette (in 0.10-2.00 mL sample volume)
Read against air (without a cuvette in the light path) or against water

Pipette into cuvettes	Blank*	Sample
sample in maleate buffer (pH ~ 6.2) solution *IV*(d) (sucrase + β-gal-ase)	0.20 mL -	0.20 mL 0.10 mL
Ensure that all of the solutions are delivered to the bottom of the cuvette. Mix the contents by gentle swirling, cap the cuvettes and incubate them at ~ 25˚C for 60 min. **Add:**		
distilled water (at ~ 25˚C) solution *IV*(b) (imidazole buffer) solution *IV*(c) ($NADP^+$/ATP)	2.10 mL 0.10 mL 0.10 mL	2.00 mL 0.10 mL 0.10 mL
Mix**, read the absorbances of the solutions (A_1) after approx. 3 min and start the reactions by addition of:		
suspension *IV*(e) (HK/G-6-PDH)	0.02 mL	0.02 mL
Mix*, read the absorbances of the solutions (A_2) at the end of the reaction (approx. 5 min). If the reaction has not stopped after 5 min, continue to read the absorbances at 2 min intervals until the absorbances remain the same over 2 min. Then add:		
suspension *IV*(f) (PGI)	0.02 mL	0.02 mL
Mix*, read the absorbances of the solutions (A_3) at the end of the reaction (approx. 8-10 min).		

* a single blank only should be performed with each set of analyses.
** for example by inversion after sealing the cuvette with a cuvette cap or Parafilm®.

professionals involved in dietary fibre research resulted in (1) 65% of the scientists supporting the definition of dietary fibre as “remnants of plant cells, polysaccharides, lignin, and associated substances resistant to hydrolysis (digestion) by the alimentary enzymes of humans”, (2) 65% of the respondents favouring inclusion of NDO and (3) 80% of the respondents supporting the inclusion of RS. In the UK, by definition dietary fibre includes only NSP.

Over the past 6 years, the importance of dietary fibre in human nutrition has been highlighted by the fact that various other official bodies or associations have become involved in the development of a dietary fibre definition. In 2000, the FNB of the Institute of Medicine of the National Academies (USA) published its definition of dietary fibre. The FNB concluded that RS and oligosaccharides that are resistant to digestion are components of dietary fibre, in agreement with the prior conclusions of the AACC definition and AOAC workshops [9]. However, the FNB divided dietary fibre into “dietary” and “functional” fibre, the two together being “total fibre”. The reason for this division is understandable, e.g. if an ingredient developer wishes to market a particular product as dietary fibre, then it should first be shown to be physiologically beneficial. However, from an analytical/regulatory standpoint, it is unmanageable. How, for example, can one distinguish between “dietary” or “functional” fibre in an oat-based breakfast cereal made from oats with extra added oat bran; or of a bread or pasta that is fortified with RS?

At the 27th session of the Codex Committee on Nutrition and Foods for Special Dietary Uses [17] a

Table 5 Values determined for D-fructose, D-glucose and ACH in a range of samples using the current ACH procedure

Sample	D-Glucose (% w/w)[a]	D-Fructose (% w/w)[a]	ACH (% w/w)[a]
Weetabix®	66.0	1.7	67.7
Cornflakes	78.0	3.1	81.1
Basmati rice	89.2	0.0	89.2
Ripe banana	32.1	24.7	56.8
Baked beans	38.4	9.6	48.0
White bread	72.3	0.1	72.4
Turnip	30.7	21.5	52.3
Onion	26.3	28.0	54.4

[a] Results on a dry weight basis. ACH values were calculated by adding D-glucose and D-fructose values.

definition of dietary fibre was proposed that also was in line with the definition proposed by AACC. However, at the 28th session of the Codex Committee on Nutrition and Foods for Special Dietary Uses [19] quite a different definition of dietary fibre was considered, namely “Dietary Fibre consists of intrinsic plant cell wall polysaccharides”. The attendees proposed that the definition of dietary fibre should be more clearly linked to fruits, vegetables and wholegrain cereals. It was stated that this definition would allow the crafting of simplistic nutritional messages. The committee concluded that to have inclusion of criteria based on the demonstration of specific physiological properties was neither appropriate nor manageable within a dietary fibre definition. The committee suggested that research should be performed to determine if carbohydrates such as RS and NDO were beneficial to health; completely disregarding all of the work already performed in this area [29–52]. The basis for measurement, and the interest in dietary fibre, is its specific physiological properties. To exclude these properties from any definition is illogical.

The FAO/WHO [18] report also states that the AOAC International procedures do not consistently measure what they were designed to measure; the problems with measurement of RS and NDO were highlighted. However, those active in research on dietary fibre methodology are well aware of these limitations, and thus the reason for the development of the AOAC methods for NDO and RS, and more specifically, the currently described integrated method for dietary fibre, RS and NDO. Dietary fibre is a mixture of carbohydrate components that display certain physiological effects. As our knowledge of these fibres increases, methodology must evolve to meet the challenges.

In appendix 2 of WHO/FAO report CRD 19, the NSP and gravimetric AOAC methods are compared with respect to performance and suitability for measurement of dietary fibre. In terms of general principles, the report concludes that the NSP procedure achieves its stated aim. However, in applying the NSP method to food products, it is impossible to determine if the measured polysaccharide is derived from intrinsic cell wall material or from an added, purified ingredient (e.g. psyllium gum, β-glucan or galactomannan).

Under the heading of “determination of dietary fibre”, the FAO/WHO report states that the definition is targeted specifically at fruits, vegetables and whole-grain products. Most consumers are well aware of the benefits of eating fruits, vegetables and cereal products. However, it is evident that not enough dietary fibre is being consumed, and this trend is associated with the rapidly increasing incidence of obesity and type 2 diabetes. Clearly, a need exists for including more dietary fibre in foods that consumers actually choose to eat. Taste, lifestyle preferences and cost factors clearly dictate against an increased consumption of fruit, vegetables and whole-grain products. It is essential that fibre components are included in the food products that consumers eat. This has spurred research into the production of a range of NSP (e.g. β-glucan, galactomannan), RS and NDO for inclusion.

In terms of potential discrepancies in methods, it is stated in the report that the AOAC enzymic-gravimetric procedures measure carbohydrates other than plant cell wall material. This, of course, is correct. The methods were designed to measure all polysaccharides other than non-resistant starch. With the NSP procedure, as stated by the authors, accurate analysis of foods containing non-cell-wall material will require the food manufacturers to provide information on the levels of other polysaccharides, so that the cell-wall-derived polysaccharides can be determined by difference. In the first instance, this information will not readily be supplied by manufacturers as this information offers them competitive advantage over other producers of similar foods. Where data are provided by manufacturers, they may not be correct, thus leading to potential errors in the final values. In contrast, the AOAC gravimetric procedure is designed to measure all non-digestible polysaccharides (including plant cell-wall polysaccharides), thus avoiding the necessity to get analytical and compositional data from suppliers.

In terms of nutritional labelling, the FAO/WHO report states that some of the carbohydrate components measured may have no effect on health, or in fact may have detrimental properties. This statement is misleading. All of the dietary fibre products that have been designed as food ingredients, and are currently marketed, have been subjected to stringent evaluation to ensure safety and physiological functionality. Extensive nutritional information is available for the most commonly used dietary fibre materials such as β-glucan [64], FOS [52, 62–65] galactomannan [64], psyllium gum [50] and RMD [65].

The FAO/WHO committee recommends that the definition of dietary fibre proposed in the 1950s be adhered to, ignoring all of the subsequent research. As our knowledge

of the physiological benefits of dietary fibre improves, we should embrace this knowledge to produce better and smarter food products.

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