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Dietary fibre analysis in foods

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Abstract: The concept of dietary fibre has been evolving over the past 20 years from a chemical to a physiological focus. This has led to changes in the definition of dietary fibre and the inclusion of carbohydrate components that previously were not considered to be dietary fibre, and, consequently, were not measured, or were partially measured. The inclusion of resistant starch and non-digestible oligosaccharides (e.g. fructo-oligosaccharides, arabino-xylosaccharides, galacto-oligosaccharides, resistant maltodextrins and Polydextrose) has required the development of improved, all-inclusive analytical methodology to service the definition. In this chapter, the current status concerning definition of dietary fibre and methodology to service this definition will be discussed. More specifically, an integrated procedure for the measurement of total dietary fibre as defined by Codex Alimentarius is described in detail.

Key words: total dietary fibre, resistant starch, non-digestible oligosaccharides, SDFP, SDFS, FOS, AXOS, Codex Alimentarius, analysis of fibre.

2.1 Introduction

> 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 (Asp, 2001).

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 (DeVries, 2004). Because dietary fibre is a multi-component

mixture, it is essential that there is a clear definition and that there is methodology to allow measurement of the defined components.

A physiological basis for the definition of dietary fibre is necessary. If it were not for the physiological effects of dietary fibre, there would be no interest in the subject on the part of researchers, consumers, regulators and manufacturers. The term 'dietary fibre' was coined and its definition refined based on observations of positive health effects related to consumption of diets rich in this component. Aspects of the definition, physiological relevance, health benefits and analytical aspects of dietary fibre have been reviewed by Champ *et al.* (2003).

The term 'dietary fibre' first appeared in 1953, and referred to the non-digestible constituents of plants that make up the plant cell wall, known to include cellulose, hemicellulose and lignin (Hipsley, 1953). 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. Later, Burkitt *et al.* (1972) recommended that individuals should increase their dietary fibre intake in order to increase their stool volume and softness. In 1974, Trowell published a definition of dietary fibre, and this definition was broadened in 1976 (Trowell *et al.*, 1976) to include all indigestible polysaccharides such as gums, modified celluloses, mucilages, oligosaccharides and pectins. The definition remained primarily physiological, identifying dietary fibre on the basis of edibility and resistance to digestion, but was broadened to reflect research findings obtained in the interim years. Some of the non-digestible polysaccharides were included because they were found to have the physiological actions attributed to dietary fibre but could not necessarily be chemically identified as having their origins in the plant cell wall. This broadened definition quickly gained widespread acceptance.

Efforts directed towards developing a method to meet these analytical requirements focused on the removal of starch and protein (Theander and Aman, 1982). 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 Fibre in Foods; Enzymatic-Gravimetric Method' (Prosky *et al.*, 1985; AOAC Official Methods of Analysis, 2010; American Association of Cereal Chemists (AACC) Method 32–05). This method employed thermostable α-amylase and amyloglucosidase (AMG) to hydrolyse starch to glucose and dextrins and protease to depolymerize protein to peptides. Ethanol is added to samples to precipitate high molecular weight soluble dietary fibre (HMWSDF) from the soluble protein and starch fragments. This method was subsequently extended to allow measurement of total, soluble and insoluble dietary fibre in foods (AOAC Official Method 991.43) (Lee *et al.*, 1992; AOAC Method 991.43). Other methods for measurement of fibre components have been developed, evaluated and subsequently approved by AOAC International. A number of these methods have recently been accepted by the Codex Committee on Methods of Analysis and Sampling (Joint Food and Agricultural Organization / World Health Organization (FAO/WHO) Food Standards Programme), as detailed in Table 2.1. In this table, dietary fibre components that are or are not measured by the particular method are shown.

Table 2.1 Methods of analysis of dietary fibre as approved by CCMAS (March 2011), showing official validation and exactly what is measured and what is not measured by the method

Method	What is measured	What is not measured	Codex type
AOAC 985.29 (AACCI 32–05.01) (Prosky) Enzymic/gravimetric (AACCI 32–05.01)	High molecular weight dietary fibre (insoluble and soluble), including: 1. Insoluble dietary fibre 2. Some resistant starch (RS2 and RS3) 3. Chemically modified starch (RS4) (overestimated) 4. Only high molecular weight soluble dietary fibre that precipitates in 4 volumes (~76%) ethanol 5. Some inulin, Polydextrose and Fibersol 2	Most resistant starch Most non-digestible oligosaccharides (e.g. FOS and galacto-oligosaccharides) Most of inulin, Polydextrose and Fibersol 2	I
AOAC 991.43 (AACCI 32–07.01) (NMKL 129, 2003) (Lee modification of Prosky.) Enzymic/gravimetric	A. Insoluble dietary fibre (including some resistant starch); and separately, B. Only high molecular weight soluble dietary fibre that precipitates in 4 volumes (~76%) ethanol, including: 1. High molecular weight soluble polysaccharides such as beta-glucan, arabinoxylan, psyllium gum, arabinogalactan 2. Some inulin, Polydextrose and Fibersol 2	Most resistant starch Most non-digestible oligosaccharides (e.g. FOS and galacto-oligosaccharides) Most of inulin, Polydextrose and Fibersol 2	I
AOAC 993.21 (Applicable to food and food products that contain more than 10% dietary fibre and less than 2% starch.)	High molecular weight dietary fibre (insoluble and soluble), including: 1. Insoluble dietary fibre 2. Some resistant starch (RS3 and RS2) 3. Chemically modified starch (RS4) (overestimated) 4. Only high molecular weight soluble dietary fibre that precipitates in 4 volumes (~76%) ethanol 5. Some inulin, Polydextrose and Fibersol 2	Non-digestible oligosaccharides (e.g. FOS and galacto-oligosaccharides) Most of inulin, Polydextrose and Fibersol 2	I

(*Continued overleaf.*)

Table 2.1 (Continued)

Method	What is measured	What is not measured	Codex type
AOAC 994.13 (AACCI 32–25.01) (NMKL 162, 1998) (Uppsala method) Enzymic/gravimetry/ Spectrophotometry Provides sugar composition and Klason lignin content	High molecular weight dietary fibre (insoluble and soluble), including: 1. Insoluble dietary fibre 2. Some resistant starch (RS2 and RS3) 3. Chemically modified starch (RS4) (overestimated) 4. Only high molecular weight soluble dietary fibre that precipitates in 4 volumes (~76%) ethanol 5. Some inulin, Polydextrose and Fibersol 2	Most resistant starch Most non-digestible oligosaccharides (e.g. FOS and galacto-oligosaccharides) Most of Inulin, Polydextrose and Fibersol 2	I
2001.03 (Suitable for all foods where resistant starches are not present.) (AACCI 32–41.01)	A. High molecular weight dietary fibre (insoluble and soluble), including: 1. Insoluble dietary fibre 2. Some resistant starch (RS3 and RS2) 3. Chemically modified starch (RS4) (overestimated) 4. Only high molecular weight soluble dietary fibre that precipitates in 4 volumes (~76%) ethanol 5. Some inulin, Polydextrose and Fibersol 2 B. Lower molecular weight soluble dietary fibre that is soluble in 4 volumes (~76%) ethanol, including: 1: Remainder of inulin, Polydextrose and Fibersol 2 2. Remainder of the non-digestible oligosaccharides (e.g. FOS and galacto-oligosaccharides)	Most resistant starch	I
2009.01 (Incubation with alpha-amylase and Amyloglucosidase (AMG) at physiological temperature.) (AACCI 32–45.01)	All dietary fibre, including: A. High molecular weight dietary fibre (insoluble and soluble), including all resistant starch B. Lower molecular weight soluble dietary fibre	Nothing	I

AOAC 991.42 (AACCI 32–20.01)	Insoluble dietary fibre	A. High molecular weight soluble fibre B. Low molecular weight soluble dietary fibre	I
AOAC 993.19	Only high molecular weight soluble dietary fibre that precipitates in 4 volumes (~76%) ethanol	A. Insoluble dietary fibre B. Lower molecular weight soluble dietary fibre	I
AOAC 995.16 (AACCI 32–23.01)	(1–3)(1–4) Beta-D-Glucans	Everything else	II
AOAC 997.08 (AACCI 32–31.01)	Fructans (Inulin, reducing and non-reducing FOS)	Everything else	II
AOAC 999.03 (AACCI 32–32.01)	Fructans (Inulin, and non-reducing FOS; reducing FOS are slightly underestimated)	Everything else	III
AOAC 2000.11 (AACCI 32–28.01)	Polydextrose	Everything else	II
AOAC 2001.02 (AACCI 32–33.01)	Trans-galacto-oligosaccharides	Everything else	II
AOAC 2002.02 (AACCI 32–40.01)	Resistant starch	Everything else	II

Other methods, namely those for the measurement of insoluble glucans and mannans of yeast cell wall, non-starch polysaccharides and an alternative method for fructo-oligosaccharides, have been accepted by Codex Alimentarius as Type IV methods (i.e. methods that have not been subjected to rigorous interlaboratory evaluation through AOAC International).

The AACC undertook a critical review of the status of dietary fibre science and definition in 1998. Over the course of a year, the committee held three workshops and provided an international website, available to all web users worldwide, to receive comments. After due deliberation, an updated definition of dietary fibre was delivered to the AACC Board of Directors for adoption in early 2000, and published (Anon, 2001):

> Dietary fibre 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 fibre includes polysaccharides, oligosaccharides, lignin, and associated plant substances. Dietary fibres promote beneficial physiological effects including laxation, and/or blood cholesterol attenuation, and/or blood glucose attenuation. (Anon, 2001)

Concurrently, in the UK, Englyst and colleagues (Englyst and Cummings, 1984; Englyst and Hudson, 1987) developed methods for the measurement of non-starch polysaccharides (NSP), based on the original work of Southgate (1969; 1982). These NSP procedures measure only non-starch polysaccharides; resistant starch (RS) and non-digestible oligosaccharides (NDO) are excluded. Starch in the sample is dissolved in hot dimethyl sulphoxide (DMSO), diluted in buffer and depolymerized with thermostable α-amylase followed by pullulanase. The recovered NSP is acid hydrolysed to monosaccharides, which are measured by high-performance liquid chromatography (HPLC) by gas liquid chromatography (after derivatization) or colorimetrically.

Several definitions of dietary fibre (DF) have appeared over the past decade. The Food Nutrition Board (FNB) of the Institute of Medicine of the National Academies (USA) (2001) defined dietary fibre as follows: 'Dietary fibre consists of non-digestible carbohydrates and lignin that are intrinsic and intact in plants. Added fibre consists of isolated, non-digestible carbohydrates that have beneficial physiological effects in humans. Total fibre is the sum of dietary fibre and added fibre.'

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

> Dietary fibre means carbohydrate polymers with a degree of polymerization (DP) not lower than 3 which are neither digested nor absorbed in the small intestine. A degree of polymerization not lower than 3 is intended to exclude mono- and

disaccharides. It is not intended to reflect the average DP of the mixture. Dietary fibre consists of one or more of:

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

At the 30th session of CCNFSDU (Codex, 2008) the committee agreed on the following definition of dietary fibre:

Dietary fibre means carbohydrate polymers[a] with ten or more monomeric units[b], which are not hydrolyzed by the endogenous enzymes in the human small intestine and belong to the following categories;

- Edible carbohydrate polymers naturally occurring in the food as consumed.
- Carbohydrate polymers which have been obtained from raw materials by physical, enzymatic or chemical means and which have been shown to have a physiological effect of benefit to health as demonstrated by generally accepted scientific evidence to competent authorities;
- Synthetic carbohydrate polymers which have been shown to have a physiological effect of benefit to health as demonstrated by generally accepted scientific evidence to competent authorities.

[a] When derived from a plant origin, dietary fibre may include fractions of lignin and/or other compounds when associated with the polysaccharides in the plant cell walls and if these compounds are quantified by the AOAC gravimetric analytical method for dietary fibre analysis. Fractions of lignin and the other compounds (proteic fractions, phenolic compounds, waxes, saponins, phytates, cutin, phytosterols, etc.) intimately 'associated' with plant polysaccharides are often extracted with the polysaccharides in AOAC 991.43 method. These substances are included in the definition of fibre insofar as they are actually associated with the poly- or oligosaccharidic fraction of fibre. However when extracted or even reintroduced into 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 from 3 to 9 monomeric units should be left to national authorities.

The European situation (Commission Directive L 285/9) is as follows:

Definition of the material constituting fibre

For the purposes of this Directive 'fibre' means carbohydrate polymers with three or more monomeric units, which are neither digested nor absorbed in the human small intestine and belong to the following categories:

— edible carbohydrate polymers naturally occurring in the food as consumed;
— edible carbohydrate polymers which have been obtained from food raw material by physical, enzymatic or chemical means and which have a beneficial physiological effect demonstrated by generally accepted scientific evidence;
— edible synthetic carbohydrate polymers which have a beneficial physiological effect demonstrated by generally accepted scientific evidence.

The fact that the AOAC procedures for measurement of dietary fibre (e.g. AOAC Methods 985.29 and 991.43) do not quantitatively measure resistant starch and, in general, measure little of the NDO (Fig. 2.1) is well known to researchers and analysts in the field. Methods have thus been developed for measurement of some specific NDO: fructo-oligosaccharides, AOAC Methods 997.08 (Hoebregs, 1997) and 999.03 (McCleary *et al.*, 2000); Polydextrose, AOAC Method 2000.11 (Craig *et al.*, 2001); Fibresol 2, AOAC Method 2001.03 (Gordon and Okuma, 2002); galacto-oligosaccharides, AOAC Method 2001.02 (de Slegte, 2002). At present, there is no specific procedure for arabino-xylo-saccharides (AXOS) (Grootaert *et al.*, 2007). Methods for the specific and accurate measurement of β-glucan, AOAC Method 995.16 (McCleary and Codd, 1991) and resistant starch, AOAC Method 2002.02 (McCleary and Monaghan, 2002; McCleary *et al.*, 2002) have also been developed and validated. The need for an integrated procedure for the measurement of dietary fibre (including resistant starch), and of all of the NDO as a group, was discussed in a methods group meeting at the conference 'Dietary Fibre 2006', Helsinki, 11–14 June 2006 (Dietary Fibre Conference, 2006). Various approaches aimed at resolving this

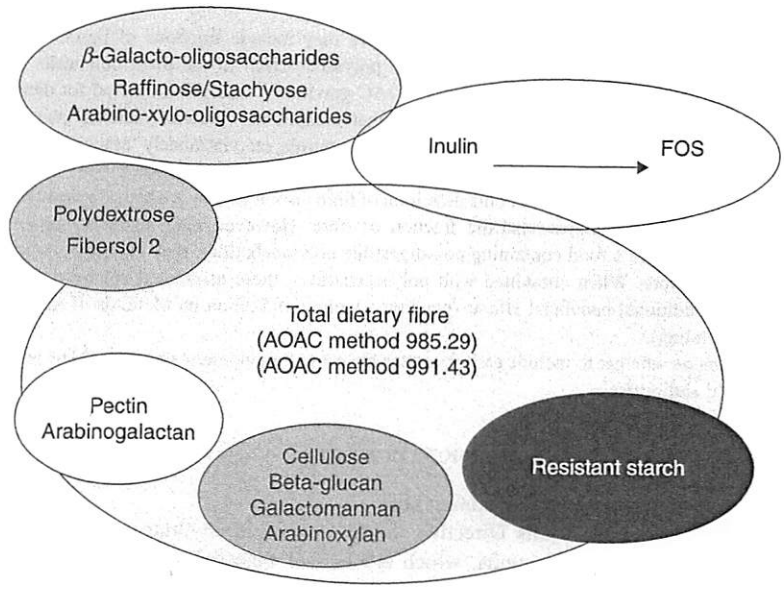

Fig. 2.1 Schematic representation of dietary fibre components measured, and not measured, by AOAC Official Methods 985.29 and 991.43. Also depicted are the problems of partial measurement of RS, Polydextrose® and resistant maltodextrins by current AOAC total dietary fibre methods. Most of the low molecular weight soluble dietary fibre (LMWSDF; galacto-oligosaccharides, fructo-oligosaccharides, etc.) are not measured. The currently described integrated total dietary fibre procedure measures all components shown, with no double counting (from McCleary *et al.*, 2010).

analytical challenge were proposed, and the method described in this chapter is one of these approaches.

An integrated method for the measurement of total dietary fibre was published in 2007 (McCleary, 2007). This method allows the accurate measurement of insoluble dietary fibre (IDF) (including resistant starch), high molecular weight soluble dietary fibre (HMWSDF; also now referred to as SDFP (soluble dietary fibre which precipitates in the presence of 76% aqueous ethanol)) and low molecular weight soluble dietary fibre (LMWSDF), also referred to as non-digestible oligosaccharides (NDO) or as soluble dietary fibre which does not precipitate in the presence of 76% aqueous ethanol (SDFS). Details of this procedure are outlined in Fig. 2.2. The use of pancreatic α-amylase at 37°C and pH 6.0 more closely simulates digestion in the human digestive tract and yields RS values in line with those obtained with AOAC Official Method 2002.02 (Table 2.2) and with results from ileostomy patients (Champ *et al.*, 2001). This method was successfully subjected to interlaboratory evaluation (McCleary *et al.*, 2010) and accepted as AOAC Official Method 2009.01. In this study, total high molecular weight dietary fibre (HMWDF) and SDFS are measured. In an Association of Official Analytical Chemists International/American Association of Cereal Chemists International (AOACI/AACCI) interlaboratory study just completed (McCleary *et al.*, 2012), the method has been evaluated for the measurement of IDF, SDFP and SDFS.

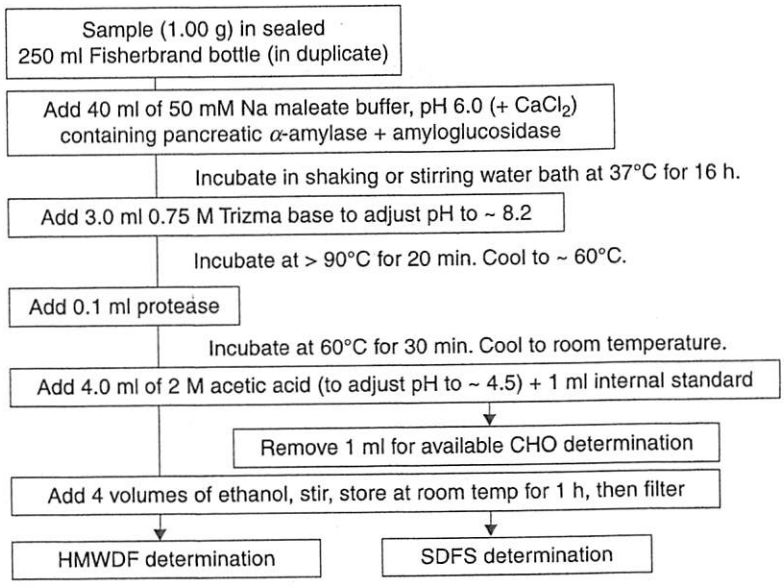

Fig. 2.2 Schematic representation of the integrated total dietary fibre (TDF) assay procedure, also showing where samples can be removed for determination of available carbohydrates.

Table 2.2 Resistant starch values determined for a number of samples using AOAC Official Methods 2002.02 and 2009.01

Sample details	Resistant starch % 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
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
Corn flakes	2.2	2.4

Notes: 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 approx. 2–3%.

2.2 An integrated procedure for the measurement of total dietary fibre, including resistant starch and non-digestible oligosaccharides

2.2.1 Principle

An integrated procedure (AOAC Method 2009.01) is described for the measurement of total dietary fibre, including RS and SDFS (i.e. NDO) of DP ≥ 3. This method combines the key attributes of AOAC Official Methods of Analysis 2002.02, 985.29, 991.43 and 2001.03. A modification of this method to allow separate measurement of IDF and SDFP is also described. Duplicate test portions are incubated with pancreatic α-amylase (PAA) and amyloglucosidase (AMG) for 16 h at 37°C in sealed 250 ml bottles in a shaking water bath while mixing with sufficient vigour to maintain continuous suspension. Alternatively, the solutions can be stirred using a 2mag Mixdrive 15® submersible magnetic stirrer with a 30 × 7 mm stirrer bar and a stir rate of 170 rpm (Fig. 2.3) (http://www.2mag.de/english/stirrer/multiple/stirrer_multiple_04_mixdrive6_15.html). During this step, non-resistant starch is solubilized and hydrolysed to D-glucose, maltose and small levels of non-hydrolysed maltodextrins by the combined action of the two enzymes. The reaction is terminated by pH adjustment followed by temporary heating. Protein in the sample is denatured and digested with protease. Specific dietary fibre fractions are measured as follows.

Fig. 2.3 Arrangements for mixing or stirring of suspensions of dietary fibre-containing samples in a 2mag Mixdrive 15® submersible magnetic stirrer with a 30 × 7 mm stirrer bar.

Insoluble dietary fibre (IDF), higher molecular weight soluble dietary fibre (soluble in water but insoluble in 76% aqueous ethanol; SDFP) and lower molecular weight soluble dietary fibre (soluble in 76% aqueous ethanol; SDFS) determination

IDF is recovered by filtration of the aqueous reaction mixture and the residue is washed, dried and weighed. SDFP in the filtrate is precipitated with ethanol or industrial methylated spirits (IMS), recovered, dried and weighed. Both the IDF and SDFP residues are corrected for protein, ash and blank values for the final calculation of the IDF and SDFP values. The aqueous ethanol filtrate from the soluble dietary fibre (SDF) fraction is concentrated, adjusted to ~ pH 4.5 and incubated with AMG (Brunt, 2011), heated to 100°C, desalted, reconcentrated and analysed by HPLC for SDFS.

Total high molecular weight dietary fibre (HMWDF) and SDFS determination

Four volumes of 95% ethanol are added to the incubation mixture and stirred. SDFP is precipitated from the incubation mixture and the suspension is filtered. The HMWDF (comprising IDF and SDFP) recovered on the crucible is washed, dried and weighed. This residue weight is corrected for protein, ash and the blank value for the final calculation. The aqueous ethanol filtrate is concentrated, incubated with AMG, heated to 100°C, desalted, reconcentrated and analysed by HPLC for SDFS.

Table 2.3 Total dietary fibre values determined for a range of samples that have been traditionally used as standards in TDF assays

Sample details	Total dietary fibre, % 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

The enzymes used in the current method are of very high purity; they are effectively devoid of contaminants active on β-glucan, pectin and arabinoxylan. SDFS such as fructo-oligosaccharides (FOS) and galacto-oligosaccharides (GOS) are not hydrolysed, and the degree of hydrolysis of Polydextrose® and Fibresol-2® is in line with the information provided by the suppliers.

To ensure the presence of the appropriate enzyme activity and absence of undesirable enzyme activity, the materials listed in Table 2.3 (available in the kit, K-TDFC from Megazyme) are analysed using the entire procedure. Each new lot of enzymes should be tested, as should enzymes that have not been tested in the previous six months.

2.2.2 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 provided the mill has sufficient air flow or other cooling to avoid overheating of samples.
2 Digestion bottles – 250 ml Fisherbrand® soda glass, wide mouth bottles with polyvinyl lined cap (cat. no. FB73219) (https://extranet.fisher.co.uk/insight2_uk/mainSearch.do?keywords=FB73219&utm_source=fisher_web&utm_medium=product_page&utm_campaign=all_product_promote; accessed 4 October 2012).
3 Fritted crucible – Gooch, fritted disk, Pyrex® 50 ml, pore size coarse, ASTM 40–60 mm, Corning® No. 32940–50C, or equivalent.
4 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 (2.2.3(15)) at room temperature for 1 h.
(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 desiccator for approximately 1 h and record mass of crucible containing Celite®.

5 Filtering flask – heavy-walled, 1–l Büchner flask (Fig. 2.4).
6 Rubber ring adaptors.– for use to join crucibles with filtering flasks (Fig. 2.4).
7 Vacuum source – vacuum pump or aspirator with regulator capable of regulating vacuum (e.g. Edwards XDS 10; single-phase 115/230V; product code: A726–01–903).
8 Water bath(s) – rotary motion (150 rpm), large-capacity (20–24 l) with covers; capable of maintaining temperature of 37 ± 1°C and 60 ± 1°C (e.g. Grant® OLS 200 shaking incubation bath). Alternatively, use a 2mag Mixdrive 15® submersible magnetic stirrer with a 30 × 7 mm stirrer bar, set at 170 rpm (Fig. 2.3).
9 Balance – 0.1 mg readability, accuracy and precision.
10 Ovens – Two, mechanical convection, set at 103 ± 2°C and 130 ± 3°C.
11 Timer.
12 Desiccator – Airtight, with silica gel or equivalent desiccant. Desiccant dried biweekly overnight in 130°C oven.

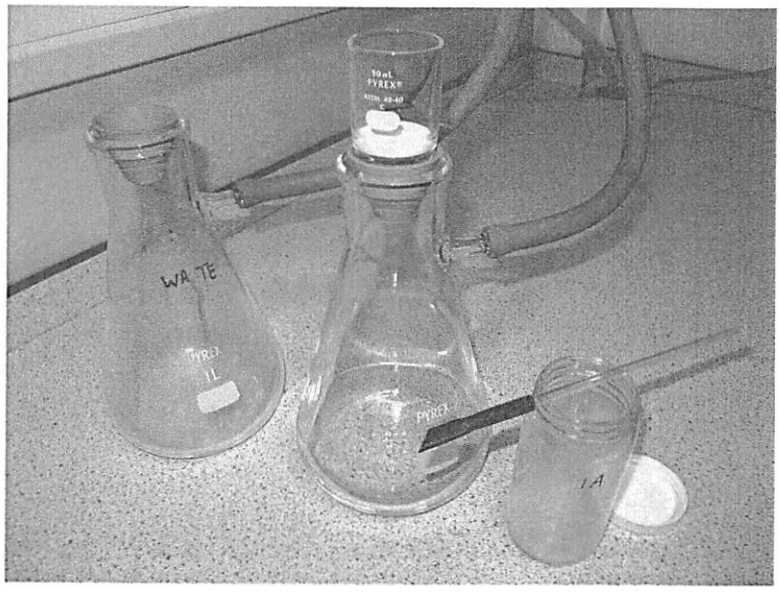

Fig. 2.4 Heavy-walled, 1 l Büchner flask with rubber ring adaptors; 250 ml Fisherbrand® soda glass, wide mouth bottles with polyvinyl lined cap; and rubber policeman spatula.

13 pH meter.
14 Thermometer – Capable of measuring to 110°C.
15 Positive displacement pipettor – e.g. Eppendorf Multipette®
   (a) with 25 ml Combitip® (to dispense 3 ml aliquots of 0.75 M Trizma® Base solution and 4 ml aliquots of 2 M acetic acid).
   (b) with 5.0 ml Combitip® (to dispense 0.3 ml aliquots of AMG and 0.1 ml of AMG and protease solutions).
16 Cylinder – Graduated, 100 ml and 500 ml.
17 Magnetic stirrers and stirring bars – (7 × 30 mm; plain magnetic stirrer bars; cat. no. 442–0269, VWR Dublin, Ireland).
18 Rubber policeman spatulas – VWR International (cat. no. 53801–008) (Fig. 2.4).
19 Muffle furnace – 525 ± 5°C.
20 Polypropylene columns – Bio-Rad, Econo-Pac™ Disposable Chromatography Columns (cat. no. 732–1010) with an Alltech One-Way Stopcock (cat. no. 211524) (Fig. 2.5).
21 Liquid chromatograph (LC) – With oven to maintain a column temperature of 90°C and a 50 µl injection loop. System must separate maltose from maltotriose.
22 Guard column (or pre-column) – Waters Guard Pak® LC pre-column inserts (Waters part no. WAT015209) or equivalent.

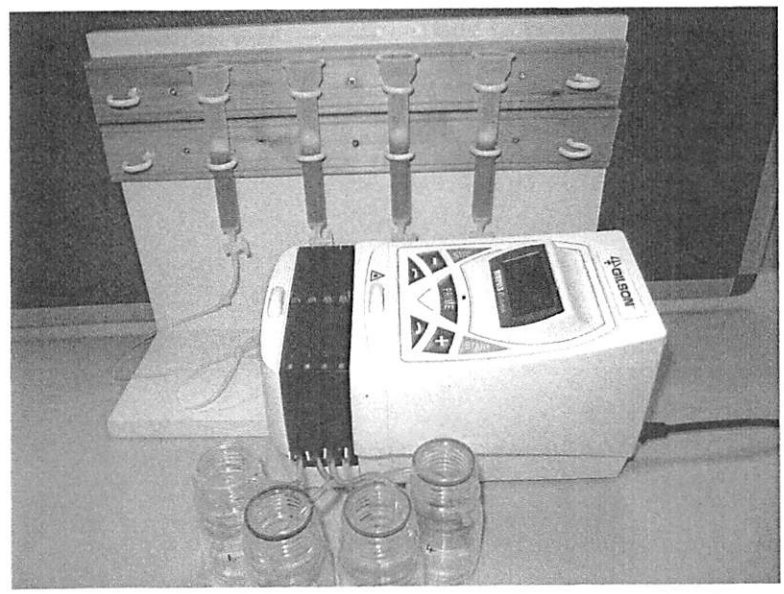

Fig. 2.5 Deionization of samples with mixed bed resin (~4 g Amberlite® FPA53 (OH^-) and ~4 g Ambersep® 200 (H^+)) in Bio-Rad, Econo-Pac® Disposable Chromatography Columns connected to a Gilson Minipuls® Evolution pump.

23 LC column – Waters Sugar-Pak® 6.5 × 300 mm column (part no. WAT085188) or equivalent. Mobile phase distilled water plus ethylene diamine tetraacetic acid disodium calcium salt ($Na_2CaEDTA$) (50 mg/l); flow rate 0.5 ml/min; column temp. 90°C; run time 30 min to assure column cleaned out.
24 Detector – Refractive index (RI); maintained at 50°C.
25 Data integrator or computer – For peak area measurement.
26 Filters for disposable syringe – Millipore Millex® Syringe Driven Filter Unit 0.45 mm (low protein binding Durapore PVDF), 25 mm or 13 mm or equivalent.
27 Filters for water – Millipore, 0.45 mm Durapore® Membrane Filters type HVLP, 47 mm.
28 Filter apparatus – To hold 47 mm, 0.45 mm filter (2.2.2(27)); to filter larger volumes of water.
29 Syringes –10 ml, disposable, plastic.
30 Syringes – Hamilton® 100 µl, 710SNR syringe.
31 Rotary evaporator – Heidolph Laborota® 4000 or equivalent.

2.2.3 Reagents

1 Ethanol (or IMS) 95% v/v.
2 Ethanol (or IMS) 78% v/v – Place 180 ml deionized water into 1 l volumetric flask. Dilute to volume with 95% v/v ethanol (or IMS). Mix.
3 Acetone, reagent grade.
4 Stock amyloglucosidase (AMG) solution (Megazyme cat. no. K-INTDF), 3300 Units/ml in 50% v/v glycerol – Solution is viscous; dispense using a positive displacement dispenser. AMG solution is stable for approx. 3 years when stored at 4°C. (Note: one unit of enzyme activity is the amount of enzyme required to release 1 micromole of D-glucose from soluble starch per minute at 40°C and pH 4.5). AMG solution should be essentially devoid of β-glucanase, β-xylanase and detectable levels of free D-glucose. Stable for > 4 years at −20°C.
5 Pancreatic α-amylase (50 units/ml)/AMG (3.4 units/ml) – Immediately before use, dissolve 0.10 g of purified porcine pancreatic α-amylase (150 000 units/g; AOAC Method 2009.01) (Megazyme cat. no. K-INTDF) in 290 ml of sodium maleate buffer (50 mM, pH 6.0 plus 2 mM $CaCl_2$ and 0.02% sodium azide) (2.2.3(14)) and stir for 5 min. Add 0.3 ml of AMG (2.2.3(4)). Stable for > 2 years at −20°C.
6 Protease (50 mg/ml; 350 tyrosine units/ml) in 50% v/v glycerol (Megazyme cat. no. K-INTDF) – Solution is viscous; dispense using a positive displacement dispenser. Protease must be devoid of α-amylase and essentially devoid of β-glucanase and β-xylanase. Use as supplied. Stable for > 3 years at 4°C.
7 LC retention time standard – Standard having the distribution of oligosaccharides (DP >3) corn syrup solids (DE 25; Matsutani Chemical

Industry Co., Ltd, Itami City, Hyogo, Japan; www.matsutani.com) analysed by LC plus maltose in a ratio of 4:1 (w/w). Dissolve 2.5 g of oligosaccharide mixture in 80 ml of 0.02% sodium azide solution (2.2.3(13)) and transfer to 100 ml volumetric flask. Pipette 10 ml of internal standard (2.2.3(8)) into the flask. Bring to volume with 0.02% sodium azide solution (2.2.3(13)). Transfer solutions to 50 ml polypropylene storage bottles®. Stable for > 1 year at room temperature. Stable for > 4 years at −20°C.

8 D-Sorbitol. (Internal standard for Sugar-Pak® column) – 100 mg/ml containing sodium azide (0.02% w/v). Weigh 10 g of analytical grade (> 99%) D-sorbitol into a 100 ml volumetric flask. Dissolve in 80 ml of 0.02% (w/v) sodium azide solution (2.2.3(13)) and adjust to volume with 0.02% sodium azide solution. Mix well. Stable for > 2 years at room temperature. Stable for > 4 years at −20°C. (Note: handle sodium azide with caution, only after reviewing material safety data sheet, using appropriate personal protective gear and laboratory hood.)

9 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; cat. no. 5767) and transfer to three separate 100 ml volumetric flasks. To each flask pipette 10 ml of internal standard (2.2.3(8)). Bring to volume with 0.02% sodium azide solution (2.2.3(13)). Transfer solutions to 100 ml Duran® bottles. Stable at room temperature for 1 year.

10 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 deionized 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 and adjust the volume to 2 l. Stable for > 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. Handle sodium azide and maleic acid with caution, only after reviewing Materials Safety Data Sheet (MSDS), using appropriate personal protective gear and laboratory hood.)

11 Trizma Base® (Sigma cat. no. T-1503), 0.75 M – Add 90.8 g of Trizma® base to approx. 800 ml of deionized water and dissolve. Adjust volume to 1 l. Stable for > 1 year at room temperature.

12 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 deionized water. Stable for > 1 year at room temperature.

13 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: do not add sodium azide to solutions of low pH. Acidification of sodium azide releases a poisonous gas. Handle sodium azide with caution, only after reviewing MSDS, using appropriate personal protective gear and laboratory hood.) Stable at room temperature for > 2 years.

14 Deionized water containing $Na_2CaEDTA$ (50 mg/l) – Weigh 50 mg of $Na_2CaEDTA$ into a 1 l Duran bottle and dissolve in 1 l distilled water. Prepare fresh weekly; filter through 0.45 mm filter (2.2.2(27)) before use.

15 Cleaning solution – Micro-90® (International Products Corp., USA (www.ipcol.com/shopexd.asp?id=15) (accessed 4 October 2012). Make a 2% solution with deionized water.
16 pH standards – Buffer solutions at pH 4.0, 7.0 and 10.0.
17 Celite® – acid-washed, pre-ashed (Megazyme G-CEL100 or G-CEL500).
18 Mixed-bed ion exchange resins for each test portion –

(a) m-1. – approx. 4 g Amberlite® FPA53 (OH^-) resin (Rohm and Haas, France S.A.S.) (see also Megazyme cat. no. G-AMBOH), ion exchange capacity 1.6 meq/ml (min) or equivalent (R-OH exchange capacity data supplied by manufacturer) and
(b) m-2. – approx. 4 g Ambersep® 200 (H^+) resin or equivalent (Rohm and Haas, France S.A.S.) (see also Megazyme cat. no. G-AMBH), ion exchange capacity: 1.6 meq/ml (minimum). Mix the two resins just prior to use and pack in column (2.2.2(20), Bio-Rad disposable chromatography column) for analysis of each test portion (see Fig. 2.5). After mixing and packing, add a small cotton wool plug and wash with 20 ml of deionized water. If using other resins and there is a concern that carbohydrates may be retained on the resin, prepare a test solution consisting of 1 ml of 100 mg/ml internal standard (2.2.3(8)) and 2.5 ml of 10 mg/ml fructo-oligosaccharides diluted to 10 ml. Proceed to step 2.2.9(1b) 'Deionization of sample'. Recovery of the internal standards and fructo-oligosaccharides should match that of the solution injected directly onto the LC (Fig. 2.6).

2.2.4 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.). De-fat per AOAC 985.29 if >10% fat. For high moisture samples (>25%) it may be desirable to freeze dry. Grind ~50 g in a grinding mill (2.2.2(1)) 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.

2.2.5 Enzyme purity

To ensure absence of undesirable enzymatic activities and effectiveness of desirable enzymatic activities, run standards (Megazyme cat. no. K-TDFC) each time the enzyme lot changes or after the enzyme has been stored for more than 6 months.

2.2.6 Enzyme digestion of samples

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

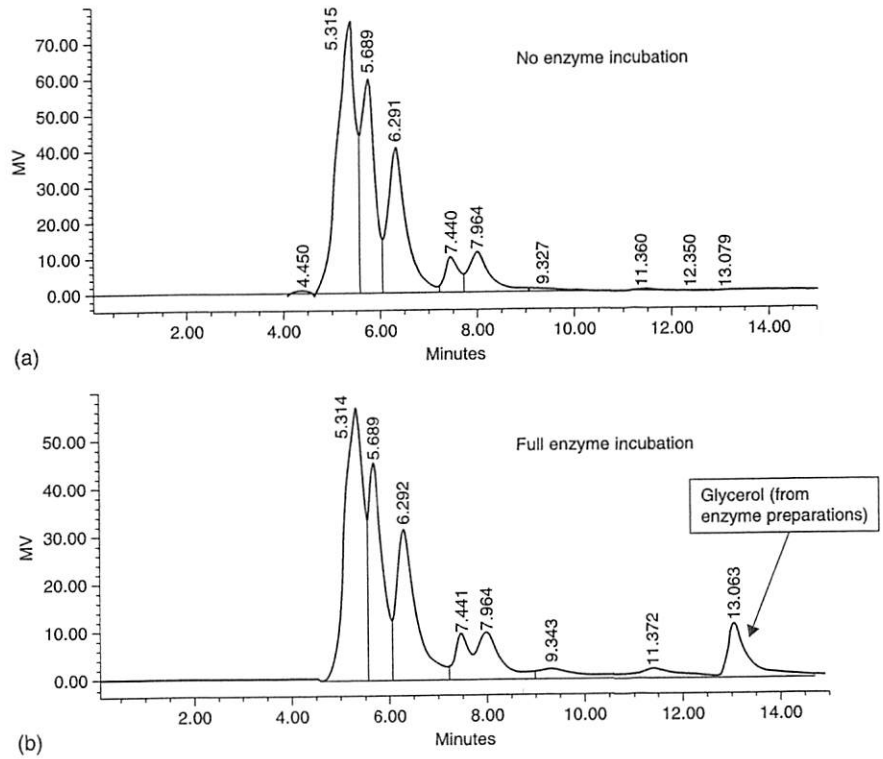

Fig. 2.6 High-performance liquid chromatography (HPLC) trace for Raftilose P-95® dissolved in water and analysed directly, compared with Raftilose P-95® recovered as NDO after running through the current integrated TDF procedure. Column: Waters Sugar-Pak® (6.5 mm × 300 mm). Solvent: distilled water containing EDTA (50 mg/l). Flow rate: 0.5 ml/min. Temperature: 90°C. (a) No enzyme incubation. (b) Full enzyme incubation.

2 Samples

(a) Weigh-duplicate 1.000 ± 0.005 g samples accurately into 250 ml Fisherbrand® soda glass, wide mouth bottles (2.2.2(2)).

(b) Addition of enzymes – Wet the sample with 1.0 ml of ethanol and add 40 ml of pancreatic α-amylase/AMG mixture (2.2.3(5)) to each bottle. Cap the bottles. Transfer the bottles to a Grant OLS 200 shaking incubation bath (or similar) (2.2.2(8)) and secure the bottles in place with the springs in the shaker frame. Alternatively, use a 2mag Mixdrive 15® submersible magnetic stirrer (2.2.2(8)) with 7 × 30 mm stirrer bars (Fig. 2.3).

(c) Incubation with pancreatic α-amylase/AMG – Incubate the reaction solutions at 37°C and 150 rpm in orbital motion in a shaking water bath (2.2.2(8)) or at 170 rpm on a 2mag Mixdrive 15® submersible magnetic stirrer (to ensure complete suspension) 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) and 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 (2.2.3(11)) 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 immediately 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 (2.2.3(6)) 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 (2.2.3(12)) to each bottle and mix. This gives a final pH of approx. 4.3.

(h) Internal standard – Add 1.0 ml of D-sorbitol internal standard solution (100 mg/ml) (2.2.3(8)) to each bottle and mix well.

3 Proceed to step 2.2.7(1) for determination of HMWDF/SDFS; or to step 2.2.8(2a) for determination of IDF/SDFP/SDFS.

2.2.7 Determination of HMWDF (IDF plus SDFP)

1 Precipitation SDFP – Preheat the sample to 60°C and add 192 ml (measured at room temperature) of 95% (v/v) ethanol (or IMS) pre-heated to 60°C. Mix thoroughly and allow the precipitate to form at room temperature for 60 min.

2 Filtration setup – Tare crucible containing Celite® (from 2.2.2(3)) to the nearest 0.1 mg. Wet and redistribute the bed of Celite® in the crucible, using 15 ml of 78% (v/v) ethanol or IMS from wash bottle. Apply suction to crucible to draw Celite® onto fritted glass as an even mat (Fig. 2.4).

3 Filtration – Using vacuum, filter precipitated enzyme digest (2.2.7(1)) through crucible. Using a wash bottle with 78% (v/v) ethanol (or IMS) (2.2.3(2)), quantitatively transfer all remaining particles to crucible. Retain filtrate and washings and proceed to step 2.2.9(1a) for determination of SDFS.

4 Wash – Using a vacuum, wash residue sequentially with two 15 ml portions of the following: 78% (v/v) ethanol (or IMS), 95% (v/v) ethanol (or IMS) and acetone.

5 Dry crucibles containing residue overnight in 105°C oven. If a forced air oven is used, loosely cover the crucibles with aluminium foil to prevent loss of dried sample.

6 Cool crucible in desiccator for approx. 1 h. Weigh crucible containing dietary fibre residue and Celite® to nearest 0.1 mg. To obtain residue mass, subtract tare weight, i.e. weight of dried crucible and Celite®.

7 Protein and ash determination – The residue from one crucible is analysed for protein, and the second residue of the duplicate is analysed for ash. Perform protein analysis on residue using Kjeldahl or combustion methods. (Caution should be exercised when using a combustion analyser for protein in the residue. Celite® volatilized from the sample can clog the transfer lines of the unit.) Use 6.25 factor for all cases to calculate mg of protein. For ash analysis, incinerate the second residue for 5 h at 525°C. Cool in desiccator and weigh to nearest 0.1 mg. Subtract crucible and Celite® weight to determine ash.
8 Determination of HMWDF – Subtract ash and protein from average residue weight and proceed to step 2.2.10 for calculation of HMWDF.

2.2.8 Determination of IDF, SDFP and SDFS

1 IDF

(a) Filtration setup – Tare crucible containing Celite® (2.2.2(4)) 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) (2.2.3(2)) from wash bottle. Apply suction to crucible to draw Celite® onto the fritted glass as an even mat (Fig. 2.4).
(b) Filtration – Using vacuum, filter the enzyme digest from step 2.2.6(2) through the crucible. Using a wash bottle with 60°C deionized water, rinse the incubation bottle with a minimum volume of water (approx. 10 ml) and use a rubber policeman (spatula) to dislodge all particles from the walls of the container. Transfer this suspension to the crucible. Wash the bottle with a further 10 ml of water at 60°C and again transfer to the crucible. Collect the combined filtrate and washings and adjust the volume to 70 ml and retain this for determination of SDFP (2.2.8(2a)) and SDFS (2.2.9(1a)).
(c) Wash – Using a vacuum, wash the residue successively with two 15 ml portions of the following: 78% (v/v) ethanol (or IMS), 95% (v/v) ethanol (or IMS) and acetone. Discard the washings.
(d) Dry crucibles containing residue overnight in 105°C oven.
(e) Cool crucibles in desiccators for approximately 1 h. Weigh crucible containing insoluble dietary fibre residue and Celite® to nearest 0.1 mg. To obtain residue mass, subtract tare weight, i.e. weight of dried crucible and Celite®. Calculate IDF; step 2.2.10.

2 SDFP

(a) Precipitation of SDFP – Pre-heat the filtrate of each sample (approx. 70 ml) to 60°C and add 280 ml (measured at room temperature) of 95% (v/v) ethanol (or IMS) (2.2.3(1)) preheated to 60°C and mix thoroughly. Allow the precipitate to form at room temperature for 60 min.
(b) Recovery of SDFP and SDFS – Proceed according to steps 2.2.7(3) to 2.2.7(8). For determination of SDFS – Proceed according to steps 2.2.9(1a) to 2.2.9(2b).

2.2.9 Determination of SDFS

Note: Proper deionization is an essential part of obtaining quality chromatographic data on SDFS. To obtain familiarity regarding the appearance of salt peaks in the SDFS chromatograms, dissolve 10 mg of sodium chloride in 9 ml of deionized water and add 1 ml of 100 mg/ml LC internal standard (2.2.3(8)) and proceed to step 2.2.9(1c) at 'Transfer the solution to a 10 ml disposable . . .'. To assure the resins being used are of adequate deionizing capacity, dissolve 10 mg of sodium chloride in 1 ml of deionized water. Add 1 ml of 100 mg/ml LC internal standard (2.2.3(8)), and proceed to step 2.2.9(1b) at 'Transfer 2 ml of this solution to the top of . . .'. The LC chromatogram of this solution should show no peaks in the time range corresponding to carbohydrates of DP3 or greater.

1 Extraction and chromatography procedure

(a) Filtrate recovery – Set aside the filtrate from one of the sample duplicates (2.2.7(3)) to use in case of spills or if duplicate SDFS data are desired. Transfer one half of filtrate (2.2.7(3)) of the other sample duplicate to a 500 ml evaporator flask and evaporate to dryness under vacuum at 60°C.

(b) Deionization of sample and AMG incubation – Add 5 ml of 150 mM HCl to the evaporator flask and swirl the flask for approx. 2 min to dissolve the sample (this adjusts the pH to ~ 4.5). Transfer the solution to a sealable polypropylene 20 ml container, add 0.1 ml of AMG (2.2.3(4)) and incubate at 60°C for 1 h. Then heat the solution at 100°C for 5 min. Transfer 2 ml of this solution to the top of the Bio-Rad disposable column containing 4 g each of freshly prepared and thoroughly mixed Amberlite FPA 53 (OH^-) (2.2.3(18)) and Ambersep 200 (H^+) (2.2.3(18)) (Fig. 2.5). Elute the column at a rate of 1.0 ml/min into a 100 ml Duran® bottle. When the sample has entered the resin, add 2 ml of distilled water to the resin and allow this to percolate in. Then add approximately 20 ml of deionized water to the top of the column and continue to elute at a rate of 1.0 ml/min. Transfer the eluate to a 250 ml round bottom rotary evaporator flask and evaporate to dryness under vacuum at 60°C. Add 2 ml of deionized water to the flask and redissolve the sugars by swirling the flask for approx. 2 min. Using a Pasteur pipette, transfer the solution to a polypropylene storage container.

(c) Preparation of samples for LC analyses – Transfer the solution to a 10 ml disposable syringe (2.2.2(29)), and filter through a 0.45 μm filter (2.2.2(26)). Use a 100 ml LC glass syringe (2.2.2(30)) to fill the 50 ml injection loop on the LC (2.2.2(21)). Perform this analysis in duplicate. Column: Waters Sugar-Pak® (6.5 × 300 mm). Solvent: distilled water containing Na_2Ca-EDTA (50 mg/l). Flow rate: 0.5 ml/min. Temperature: 90°C.

(d) Determine the response factor for D-glucose – Since D-glucose provides an LC refractive index (RI) response equivalent to the response factor for the non-digestible oligosaccharides that make up SDFS, the LC is calibrated using D-glucose, and the response factor is used for determining

the mass of SDFS. Use a 100 µl LC syringe to fill a 50 µl injection loop for each standard D-sorbitol/D-glucose solution. Inject in triplicate.

2 Obtain the values for the peak areas of D-glucose and internal standard from the three chromatograms. The reciprocal of the slope obtained by comparing the ratio of peak area of D-glucose / peak area of D-sorbitol internal standard (y-axis) to the ratio of the mass of D-glucose / mass of D-sorbitol (x-axis) is the 'response factor'. Determine the average response factor (typically 0.97 for D-sorbitol).

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

where:

PA-IS = peak area internal standard (D-sorbitol);
PA-Glu = peak area D-glucose;
Wt-Glu = mass of D-glucose in standard;
Wt-IS = mass of D-sorbitol in standard.

(a) Calibrate the area of chromatogram to be measured for SDFS – Use a 100 ml LC syringe (2.2.2(30)) to fill the 50 ml injection loop with retention time standard (2.2.3(7)). Inject in duplicate. Determine demarcation point between DP 2 and DP 3 oligosaccharides (disaccharides maltose versus higher oligosaccharides) (Fig. 2.7).

(b) Determine peak area of SDFS (PA-SDFS) and internal standard (PA-IS) in chromatograms of sample extracts – Inject sample extracts (2.2.9(1c)) on LC. Record area of all peaks of DP greater than the DP2/DP3 demarcation point as PA-SDFS. Record the peak area of internal standard as PA-IS.

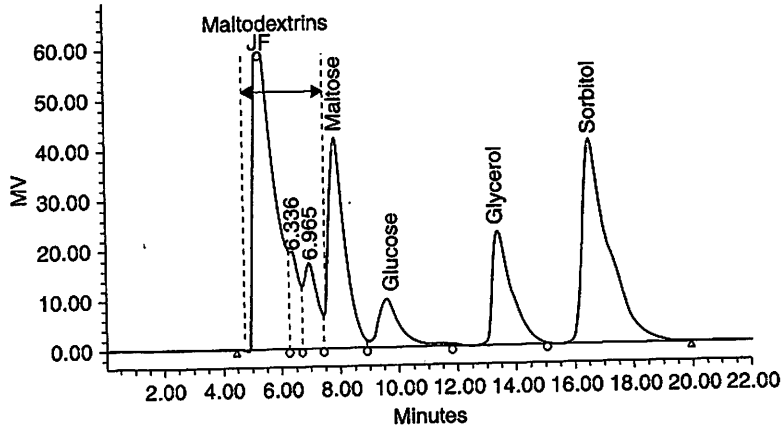

Fig. 2.7 HPLC chromatography of maltodextrins, maltose, glycerol and D-sorbitol on a Waters Sugar-Pak® column (see Fig. 2.6 for conditions).

2.2.10 Calculations for HMWDF, IDF and SDFP

Blank (B) determination (mg):

$$= (BR_1 + BR_2)/2 - P_B - A_B$$

where:

BR_1 and BR_2 = 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, IDF or SDFP (mg/100 g)

$$= \{[(R_1 = R_2)/2 - P_B - P_A - B] / (M_1 + M_2)/2\} \times 100$$

where:

R_1 = residue mass 1 from M1 in mg;
R_2 = residue mass 2 from M2 in mg;
M_1 = test portion mass 1 in g; M_2 = test portion mass 2 in g;
P_A = ash mass from R_1 in mg: P_B = protein mass from R_2 in mg.
HMWDF (%) = HMWDF (mg/100 g)/1000
IDF (%) = IDF (mg/100 g)/1000
SDFP (%) = SDFP (mg/100 g)/1000

2.2.11 Calculations for SDFS

SDFS (mg/100 g)

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

where:

Rf is the response factor.

Wt-IS is weight in mg of internal standard contained in 1 ml of internal standard solution pipetted into sample mixture (100 mg).

PA-SDFS is the peak area of the SDFS.

PA-IS is the peak area of the internal standard (D-sorbitol).

M is the test portion mass (M_1 or M_2) in grams of the sample whose filtrate was concentrated and analysed by LC.

2.2.12 Calculation of integrated TDF

Integrated TDF (%) = HMWDF (%) + SDFS (%)

2.3 Updates of the original integrated total dietary fibre procedure

The original integrated procedure for the measurement of total dietary fibre (TDF) was published in 2007 (McCleary). Since then, efforts have been made to simplify the method and to allow the processing of a larger number of samples

in a given time. Several aspects of the assay have been re-evaluated, including the incubation conditions, desalting format and choice of internal standard and LC chromatographic columns.

2.3.1 Incubation conditions

The incubation conditions for the integrated dietary fibre procedure are modelled on those used in AOAC Official Method 2002.02 (resistant starch). In that procedure, incubations were designed to give hydrolysis of non-resistant starch only. The method was developed using a set of resistant starch-containing samples that had been characterized through studies with ileostomy patients. In contrast to the resistant starch method, in the integrated dietary fibre method it is necessary to include an incubation step at approx. 100°C for the denaturation of protein; otherwise, protein is not degraded by protease. During this step, some of the resistant starch is solubilized. To ensure that none of this is depolymerized by α-amylase and AMG, these enzymes must be inactivated or denatured before the resistant starch is solubilized. This is achieved using a combination of pH and temperature conditions. As a result, most of the solubilized resistant starch precipitates from solution on addition of ethanol (to 76% concentration) and recovered as HMWDF. Lower degree of polymerization starch fragments that are resistant to hydrolysis by AMG and pancreatic α-amylase in the assay, but are not resistant to the α-glucosidases in the small intestine, are removed by incubating the SDFS fraction with much higher levels of AMG before desalting the SDFS sample.

To ensure that resistant starch is not solubilized during the 16 h incubation period, reactions were initially performed in bottles in which the contents were suspended by rotary shaking in a shaking water bath. More recently, it has been found that stirring of the contents, either with a suspended magnetic stirrer bar or with a stirrer bar in the reaction bottle, gave similar values for most samples. The results obtained for three types of starch are shown in Fig. 2.8. Dietary fibre values obtained for regular maize starch and high amylose maize starch were the same. With native potato starch granules, similar values were obtained when samples were incubated in the shaking water bath or were stirred with a suspended stirrer. When a stirrer bar is added into the incubation bottle, values for potato starch drop dramatically. This is thought to be due to the physical damage caused to the starch granules by the stirrer bar. This type of stirring had little or no effect on the determined resistant starch (and dietary fibre) in kidney beans, green bananas and various high amylose starch samples. Consequently, stirring of the reaction mixture in bottles with a stirrer bar can be performed using a device such as a Mixdrive 15® submersible magnetic stirrer.

2.3.2 Internal standards

Since AOAC Official Method 2001.03 'Total dietary fiber in foods containing resistant maltodextrins – enzymatic-gravimetric method and liquid chromatography determination" (Gordon and Okuma, 2002) employs gel permeation

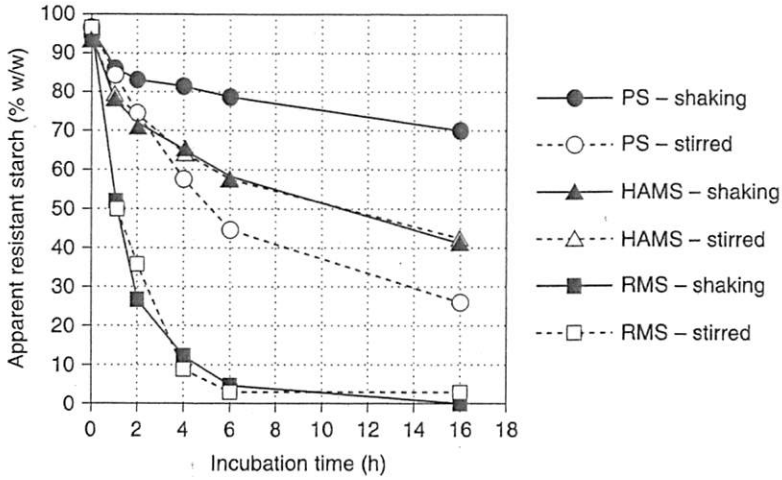

Fig. 2.8 Effect of shaking, suspended stirring and stirring with a magnetic stirrer bar added to the incubation bottle on the time course of hydrolysis of regular maize starch (RMS), high amylose maize starch (HAMS) and native potato starch (PS).

chromatography (TSK-GEL G2500PWXL®) rather than ion exchange as with the Waters Sugar-Pak column, attempts were made to find an internal standard that would work in both systems. Glycerol was considered to be non-ideal because glycerol occurs in a lot of processed food products and is also used as a stabilizer in the enzymes employed in the incubations. D-Sorbitol works well with the Sugar-Pak column, but it chromatographs with D-glucose on the TSK gel permeation columns. Numerous other compounds including sugars, sugar alcohols, diols and glycols were evaluated. Of these, 1,2-pentanediol, 1,5-pentanediol, diethylene glycol and triethylene glycol were the best based on chromatography in the two systems. However, 1,2-pentanediol showed significant loss in handling. Of the remainder, diethylene glycol was the preferred compound. However, even with this compound, if the samples were taken to dryness on rotary evaporation, a small percentage of the diethylene glycol was lost (presumably coating to the inside of the flask). Of the potential internal standards with acceptable chromatography properties, the only one suitable for the Waters Sugar Pak® column was D-sorbitol.

D-Sorbitol itself is not an ideal internal standard. The commercial fibre product Polydextrose® contains approx. 2% D-sorbitol and it is thought that this will interfere with analysis of products containing this product. However, it is unlikely that any food product will contain more than 10% Polydextrose®, in which case the level of D-sorbitol is approx. 0.2%, which will have insignificant effect on the assay. Also, if D-sorbitol is thought to be present in the sample, analysis can be performed without addition of the internal standard or, alternatively, calculations can simply be performed using the external standard assay format. Also, in such cases, the use of diethylene glycol as the internal standard could be considered (Fig. 2.9), knowing that samples should not be taken to dryness on rotary evaporation.

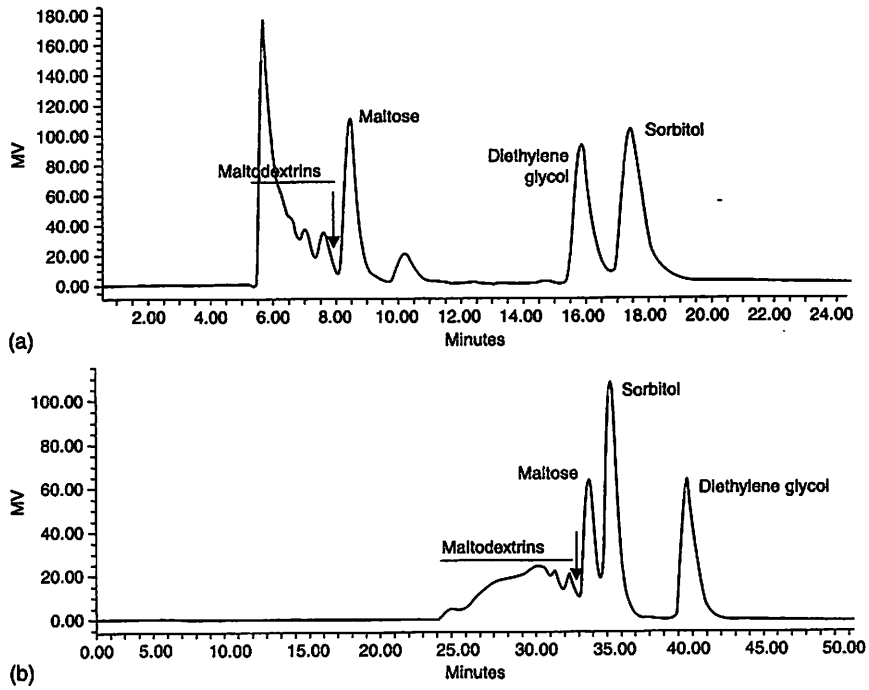

Fig. 2.9 Chromatography of a mixture of maltodextrins, maltose, diethylene glycol and D-sorbitol on: (a) a Waters Sugar-Pak® (6.5 × 300 mm, part no. WAT085188) column. Solvent: distilled water containing EDTA (50 mg/l); flow rate: 0.5 ml/min; temperature 90°C; or (b) on two TSK® gel filtration columns (G2500PWXL) in series. Solvent: distilled water; flow rate 0.5 ml/min; temperature 80°C. The arrows show demarcation between DP 2 (maltose) and DP 3 (higher maltodextrins).

2.3.3 Desalting of samples for HPLC

In the original integrated dietary procedure, the desalting conditions recommended in AOAC Official Method 2001.03 were employed, in which 25 g each of Amberlite cation and anion exchange resins were mixed and packed into a chromatography column. Subsequently, the amount of resin was halved, along with sample size. More recently, with the use of an internal standard that was completely recovered (D-sorbitol), it has been possible to reduce the amount of sample handled, along with the amounts of desalting resins to approximately 20% of that originally recommended. There are several advantages in this modification. First, all evaporations are reduced to 20%, which is a major time saving. Second, cheap, easy to use plastic columns (Bio-Rad, Econo-Pac™ Disposable Chromatography Columns) can be used. These are large enough to hold the 8 g of mixed bed resin and to accommodate the volume (20 ml) of deionized/distilled water used to elute the sugars from the column (Fig. 2.5). Essentially all sugar is eluted with this volume of water (Fig. 2.10). Also, the rates of elution of the sugars in the sample and the D-sorbitol internal standard are the same.

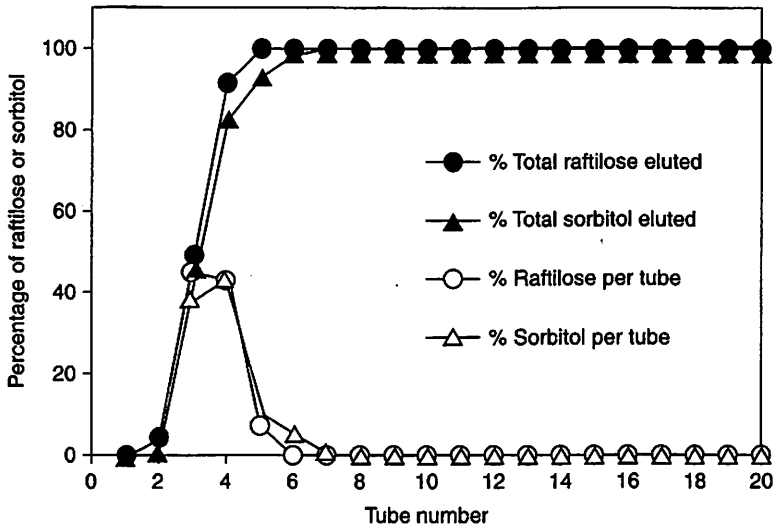

Fig. 2.10 Elution of D-sorbitol and fructo-oligosaccharides from a mixed bed resin column (Amberlite® FPA53 (OH–) plus Ambersep® 200). Note that the rate of elution of the D-sorbitol and fructo-oligosaccharides is the same and that essentially all carbohydrate is eluted with 20 ml of water.

Alternatively, samples can be desalted/de-ashed using cation and anion exchange guard columns, H^+ and CO_2^{3-} forms, respectively (BioRad Labs Catalogue # 125–0118) (Post *et al.*, 2010). In this case, the aqueous ethanolic solution is concentrated by rotary evaporation, redissolved in distilled water, adjusted to volume, clarified and injected directly onto the HPLC via the de-ashing guard columns. This procedure simplifies sample preparation for LC, and it is possible to monitor exhaustion of the de-ashing columns. A major consideration is the cost of the disposable de-ashing guard columns and the need for a separate pump for the HPLC.

2.3.4 Analysis of difficult samples

For most samples analysed to date, chromatography on a Waters Sugar-Pak® column gives effective separation of disaccharides and trisaccharides. The one exception noted is the fructosyl-trisaccharide (F3) (inulotriose) obtained on depolymerization of inulin. From Fig. 2.11, it can be seen that this compound elutes after the disaccharides sucrose and maltose, and partially overlaps them. In fact, it chromatographs at the same point as lactose. In handling oligosaccharide mixtures containing this compound, there are two possible options. Compounds can be separated using TSK gel permeation chromatography (TSK-GEL G2500PWXL®) rather than ion exchange as with the Waters Sugar-Pak®, but without the option of using D-sorbitol as an internal standard. Alternatively, an aliquot of the sample prepared for HPLC can be incubated with a mixture

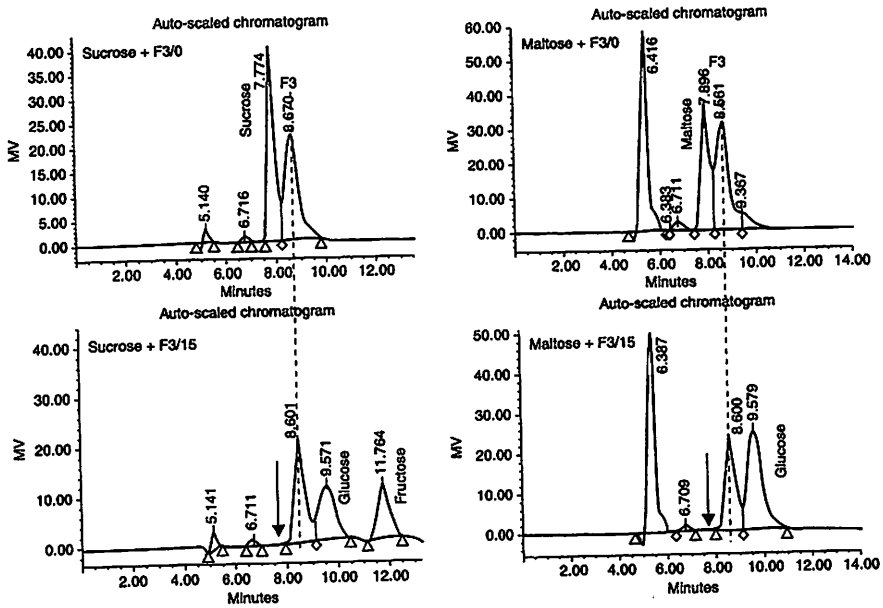

Fig. 2.11 Hydrolysis of sucrose and maltose with thermostable α-glucosidase. One millilitre of a mixture of F3 (5 mg/ml) with either sucrose or maltose (5 mg/ml) was incubated with 0.1 ml of thermostable α-glucosidase (500 U/ml) in 10 mM sodium maleate buffer (pH 6.0) at 50°C for 15 min. Reaction tube was incubated at 100°C for 5 min. The solution was centrifuged in a microfuge at 12 000 rpm for 5 min and the sample directly applied to the HPLC column.

of thermostable α-glucosidase (to hydrolyse the sucrose and maltose) and β-galactosidase (to hydrolyse lactose) and chromatographed again to measure F3 (by reference to the D-sorbitol internal standard). This value can simply be added to the value obtained for the oligosaccharide mixture of DP $\geq$ 3 obtained for the sample before α-glucosidase/β-galactosidase treatment.

Samples containing highly viscous/gelatinous dietary fibre (e.g. psyllium gum) are very difficult to filter through crucibles. Such samples may be better handled by recovering the various fractions by high-speed centrifugation. Work in this area is urgently required.

2.4 Interlaboratory evaluation of integrated total dietary fibre procedures

The integrated total dietary fibre assay procedure has been subjected to two major interlaboratory studies under the auspices of AOAC International. In the first study (McCleary *et al.*, 2010), total HMWDF and SDFS were measured. HMWDF was not fractionated into IDF and SDFP. On the basis of the results from this study, the method was accepted as AOAC Official Method 2009.01. In the second

Table 2.4 Statistical data for total dietary fibre (HMWDF plus LMWSDF) determined using the modification of AOAC Official Method 2009.01

Sample/ Parameter	Cabbage	Apple flakes	Chocolate	Biscuits	Cookies	Peanuts	Oat bran	Bread
# of Labs	15	15	15	15	14	13	14	13
Mean %	29.90	10.45	26.55	11.79	21.37	16.39	23.71	18.40
S_r	0.86	0.47	1.39	0.49	0.52	1.41	0.87	0.64
S_R	2.05	0.95	2.74	1.10	1.72	2.37	3.14	1.56
RSD_r	2.88	4.51	5.25	4.17	2.43	8.60	3.65	3.47
RSD_R	6.85	9.11	10.31	9.30	8.04	14.48	13.23	8.47
HORRAT	2.85	3.24	4.22	3.37	3.19	5.51	5.33	3.28

Source: McCleary *et al.*, 2012.

Note: Sr = within laboratory repeatability; SR = between laboratory variability; RSDr = within laboratory relative repeatability; RSDR = between laboratory relative variability; HORRAT = The ratio of the reproducibility standard deviation calculated from the data to the predicted relative standard deviation.

study, IDF, SDFP and SDFS were measured separately (McCleary *et al.*, 2012). On the basis of the results from that study, the method has recently been accepted as AOAC Official Method 2011.25. A summary of the results for TDF is shown in Table 2.4. The statistics compare favourably with those obtained for other interlaboratory evaluations of dietary fibre methods (Table 2.5) (McCleary *et al.*, 2010). Of particular interest is the fact that TDF values determined by summing HMWDF and SDFS are very similar to those obtained by summing IDF, SDFP and SDFS (Table 2.6).

2.5 Progress in acceptance of dietary fibre methodology by Codex Alimentarius

At the 30th session of CCNFSDU (2008), the Committee agreed on a definition of dietary fibre (detailed above). However, the Committee also agreed on the establishment of an Electronic Working Group (eWG) led by the Delegation of France, open to all Codex members. The specific role of this eWG was to: a) review and update, as appropriate, the list of methods available for dietary fibre analysis, taking into account the new provisions in the draft definition of dietary fibre that would require the selection of methods of analysis, and possible information on new available methods; b) consider how the results from different methods specific to different types of dietary fibre could be combined together to arrive at the total dietary fibre content in a food; c) evaluate the performance of methods in measuring different types of dietary fibre; d) make recommendations for methods of analysis for dietary fibre in different food matrices; e) consider the footnote in the accepted definition that relates to oligosaccharides of degree of polymerization (DP) of 3–9, and to prepare a recommendation as to its revision with regard to the methods of analysis, if necessary.

Table 2.5 Statistical details for various dietary fibre methods run through AOAC International interlaboratory evaluations

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	4.58–10.90	1.44–3.74
2009.01	Total Dietary Fiber in Foods	0.41–1.43	1.65–12.34	1.18–5.44	4.70–17.97	1.91–6.49

Source: McCleary *et al.*, 2012.
Notes: *Samples that were not dried and/or desugared only.

Table 2.6 Collaborative study data for total dietary fibre (% TDF) as measured directly versus the sum of IDF and SDF reported by laboratories who ran both methods

Sample Lab #	Cabbage		Apple flakes		Chocolate		Biscuits		Cookies		Peanuts		Oat bran		Bread	
1 Direct	28.51	29.58	9.78	9.51	25.80	25.30	11.51	11.52	20.50	19.85	14.81	14.11	20.90	23.16	17.69	17.70
1 Sum	28.90	27.82	9.87	9.80	27.05	28.32	11.78	12.02	21.03	20.22	17.12	17.35	22.90	23.04	17.25	17.81
2 Direct	27.42	27.86	10.09	9.53	23.49	23.06	11.75	11.71	19.10	19.02	15.25	15.71	19.65	20.23	17.13	17.46
2 Sum	27.93	29.32	10.61	10.04	24.01	23.56	12.26	12.21	19.62	19.53	15.76	16.85	20.17	20.74	17.64	17.97
3 Direct	27.56	27.32	9.74	10.10	23.69	24.84	8.49	8.89	20.98	22.15	14.62	15.69	22.48	22.68	17.50	17.50
3 Sum	28.87	28.75	9.39	9.95	25.97	29.51	10.06	10.23	24.15	24.94	19.63	21.29	34.48	21.49	22.79	20.47
4 Direct	28.07	27.76	11.89	12.88	27.88	29.34	13.13	14.01	23.10	21.77	29.09	27.29	23.25	20.89	17.10	13.19
4 Sum	27.07	26.45	9.67	10.10	21.97	21.00	12.02	13.31	19.24	18.31	13.95	13.41	20.52	17.38	16.22	16.43
5 Direct	27.79	29.04	9.02	8.55	23.24	23.30	10.12	10.40	19.75	19.97	14.68	14.17	20.74	19.90	11.86	11.73
5 Sum	28.12	29.59	9.24	9.02	23.34	23.54	10.25	10.01	20.63	20.67	*	*	20.40	20.30	12.15	11.53

Source: McCleary *et al.*, 2012.
Notes: *Laboratory reported no result for this sample.

In their draft document (Alinorm, 2009), the eWG noted that the Official AOAC methods are widely accepted globally for general labelling of nutrient content in foods as well as for health and nutrition claims. The AOAC methods are designed to be accurate, cost effective, and reproducible in various analytical environments on which industry relies. They are the most studied and validated methods available for the quantification of food components. Their use in routine analysis presents no insurmountable difficulty. These methods have passed the rigour of scientific substantiation to achieve the status of reference methods. The eWG also noted that (at that time) no one AOAC validated method could measure all non-digestible carbohydrates in foods. AOAC 991.43 (Lee *et al.*, 1992) is one of the most widely used 'total' dietary fibre methods. Both this method and AOAC 985.29 (Prosky *et al.*, 1985) will measure insoluble polysaccharides and soluble high molecular weight components (i.e. those that are precipitated by alcohol (SDFP)). However, neither fully measures the resistant starch fraction, nor do they recover the non-digestible oligosaccharide components included in the definition of dietary fibre. They quantify only part of the total resistant starch, inulin, Polydextrose (Craig *et al.*, 2000), fructo-oligosaccharides and resistant maltodextrin, all of which have relevant physiological functions. Furthermore, some oligosaccharides are not measured at all. The eWG also noted that, due to the complexity of the molecular structure of fibres, additional AOAC methods were subsequently developed to validate labelling declarations and claims by measuring specific dietary fibre components in foods that have been shown to exert physiological benefit. Maintaining these methods (e.g. AOAC 999.03 (McCleary *et al.*, 2000) for fructans) has a number of advantages. By focusing on one component the method is more specific, resulting in higher specificity and accuracy needed to detect fibre present in food products. Equally important, these component-specific methods facilitate routine, cost-effective analysis.

The eWG concluded (draft document) that the NSP method does not accurately quantify total dietary fibre. It is inappropriate as a routine technique given its inability to support the now agreed upon Codex definition of dietary fibre. Methods measuring NSP alone give lower estimates than methods for total dietary fibre in foods containing resistant starch, resistant oligosaccharides and/or lignin. The eWG did not recommend the inclusion of methods where there is as yet no publication about protocol and relevant validation data.

The eWG also noted that the definition encompasses a range of different types of carbohydrate polymers that are recovered to varying extents by different analytical methods. This creates potential problems of double accounting when a carbohydrate fraction is partially or completely measured by more than one method. Examples of this are high molecular weight inulin, which, in addition to being measured specifically by enzymatic–chemical fructan methods, is also partially recovered in the residue of enzymatic–gravimetric methods (Quemener *et al.*, 1993; 1997). The enzymatic–gravimetric methods AOAC 991.43 and 985.29 also recover some, but not all, resistant starch (McCleary and Rossiter, 2004), which can create a double accounting problem if these data are then combined with that obtained by a separate specific determination of resistant starch. There is also the potential for obtaining a lower than expected value if there

is under-recovery of a specific fraction by particular methods. The high degree of specificity associated with most direct chemical methods generally means that the problems of combining results from different methods are diminished.

The eWG noted (draft document) that the lack of a validated procedure to combine AOAC methods to determine total fibre content has repeatedly raised concerns during the lengthy process to finalize the definition of dietary fibre. It also noted that, in response to this gap in methodology, a new integrated method of analysis of total dietary fibre has been developed by McCleary (2007) which measures total dietary fibre (including resistant starch), non-digestible oligosaccharides and available carbohydrates. This new integrated method is based principally on existing official AOAC methods 2002.02 and 991.43 and AOAC method 2001.03 (Gordon and Okuma, 2002). A process similar to that described in AOAC Official Method 2001.03 allows the measurement of non-digestible oligosaccharides in the range from DP 3 to approx. DP 10.

The eWG concluded that 'this new integrated method provides a path forward for analysing the full range of dietary fibres included in the scope of the Codex definition, in a manner that better reflects overall the fiber that is physiologically relevant. This method is in the stage of collaborative study analysis and is likely to achieve AOAC approval'. In addition, the eWG suggested that the Committee should consider the inclusion of the new method of analysis for total dietary fibre (McCleary, 2007), once AOAC validation has been completed.

In the 34th session of CCNFSDU (Geneva, Switzerland, 4–9 July 2011), the outcome of the 32nd meeting in Santiago, Chile (1–5 November 2010) was reported as follows:

> **Method of analysis of dietary fibre**
>
> 14. The Committee recalled that the 31st Session of CCMAS had indicated that most of the methods of analysis for dietary fibre were empirical and some of them might be overlapping, and therefore had agreed that they could be endorsed as Type IV in order to make them available as Codex methods and asked the CCNFSDU to define their scope more precisely.
>
> 15. The Committee agreed to change the provisions for six general methods of analysis to describe them more precisely and proposed them as Type I methods. Regarding eight methods that measure individual specific components, the Committee agreed to propose them as Type II methods (one of these was subsequently changed to a Type III method). Regarding the three 'other methods', the Committee agreed to propose that they should be maintained as Type IV methods (See Appendix VI). Some delegations indicated that they were unable to comment at this stage and would make their comments to the CCMAS.
>
> 16. In reply to the proposal of CCMAS to delete the AOAC 2001.03 method, the Committee agreed to keep it because it was applicable when resistant starches are not present and AOAC 2009.01 was applicable to food that may, or may not, contain resistant starches.

These agreements are summarized in Table 2.1 (see page 27).

After many years of debate within the working groups of Codex Alimentarius, finally, a consensus opinion on a definition of dietary fibre has been agreed. With

the recent agreements on dietary fibre methodology and Codex Alimentarius typing (CCMAS, Budapest, March 2011), a clear guideline has been given to food manufacturers, analysts and regulators.

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