7 Measurement of Resistant Starch and Incorporation of Resistant Starch into Dietary Fibre Measurements

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7.1 INTRODUCTION

Resistant starch (RS) is that portion of the starch that is not broken down and absorbed in the small intestine of humans. It enters the large intestine, where it is partially or wholly fermented. The presence of a starch fraction resistant to enzymic hydrolysis was first recognized by Englyst *et al.* (1982) during their research on the measurement of non-starch polysaccharides.

Several *in vivo* approaches have been adopted for the measurement of resistant starch, including: the hydrogen breath test; direct collection of ileal effluent from patients (ileostomy patients) who have had the colon removed; and direct collection of the ileal effluent from healthy subjects using a long triple lumen tube (Champ *et al.*, 2001). Of these, the ileostomy model is considered to be the best, but not necessarily perfect.

It is generally accepted that any *in vitro* method used to measure resistant starch must give values in line with those obtained with ileostomy patients. Berry (1986) modified the *in vitro* method of Englyst *et al.* (1982) to mimic physiological conditions more closely. Incubations were performed at 37 °C. Pancreatic α-amylase and pullulanase was again employed, but the initial heating step at 100 °C was omitted. Using this method, the measured resistant starch contents of samples were much higher than those previously obtained. This was subsequently confirmed by Englyst & Cummings (1985, 1986, 1987) through studies with healthy ileostomy subjects.

Resistant Starch: Sources, Applications and Health Benefits, First Edition.
Edited by Yong-Cheng Shi and Clodualdo C. Maningat.

These authors (Englyst *et al.* 1992) also divided RS into three classes, namely:

- RS1: physically trapped starch as found in coarsely ground or chewed cereals, legumes, and grains.
- RS2: resistant starch granules or non-gelatinized starch granules which are highly resistant to digestion by α-amylase until gelatinized, e.g. uncooked potato, green banana and high-amylose starch).
- RS3: retrograded starch polymers (mainly amylose), which are produced when starch is cooled after gelatinization or during heat-moisture treatment on annealing of starch granules (during which the starch is not gelatinized).

A fourth type of resistant starch (RS4; chemically modified starch) was introduced by Brown *et al.* (1998). This starch, unlike RS1, RS2 and RS3, contains additional chemical groups. Englyst *et al.* (1992) also reported on a method for the measurement of readily digested starch (RDS), slowly digested starch (SDS) and resistant starch (RS). In this method, resistant starch is calculated by subtracting the sum of RDS plus SDS from total starch. Although the method can yield useful information, it is very laborious and gives poor reproducibility without extensive training of the analyst (Champ *et al.*, 2001). Accuracy is severely hampered by the fact that, with samples containing high levels of starch with low resistant starch content, one large analytical value is subtracted from another large value. In fact, the errors in the measurement may be as large as the resistant starch value, e.g. materials with approximately 70% starch and 2% resistant starch.

By the early 1990s, the physiological significance of RS was fully realized. Several new or modified methods for its measurement were developed during the European Research Program, EURESTA (Englyst *et al.*, 1992; Champ, 1992). The Champ (1992) method, was based on modifications to the method of Berry (1986), and gave a direct measurement of RS. Basically, sample size was increased from 10 mg to 100 mg, the sample was digested with pancreatic α-amylase only, and incubations were performed at pH 6.9 (pH 5.2 was used by Englyst *et al.* (1982, 1992) and Berry (1986)). RS determinations were performed directly on the pellet.

Muir & O'Dea (1992) developed a procedure for RS in which samples were chewed, treated with pepsin and then with a mixture of pancreatic α-amylase and amyloglucosidase (AMG) in a shaking water bath at pH 5.0, 37 °C for 15 hours. The residual pellet (containing RS) was recovered by centrifugation and washed with acetate buffer by centrifugation, and the RS was digested by a combination of heat, DMSO and thermostable α-amylase treatments.

Other methods for RS determination were developed by Faisant *et al.* (1995), Goni *et al.* (1996), Akerberg *et al.* (1998) and Champ *et al.* (1999). These modifications included changes in enzyme concentrations employed, types of enzymes used (all used pancreatic α-amylase, but pullulanase was removed, and in some cases replaced by AMG), sample pre-treatment (chewing), pH of incubation and the addition (or not) of ethanol after the α-amylase incubation step (Champ *et al.*, 1999).

7.2 DEVELOPMENT OF AOAC OFFICIAL METHOD 2002.02

While significant steps were made in the development of *in vitro* methods for the measurement of resistant starch during the 1990s, none of these methods were successfully subjected to interlaboratory evaluation. This prompted McCleary & Monaghan (2002) to look at each of these methods in detail, to evaluate all of the parameters involved and to identify sources of variability. The ultimate aim was to develop a procedure that gave values in line with those obtained with ileostomy patients, but also a method that could survive the rigors of interlaboratory evaluation. Parameters investigated included:

a) incubation conditions (shaking/stirring, pH, temperature, time);
b) level of pancreatic α-amylase employed;
c) level of AMG employed;
d) the importance of protease pre-treatment;
e) procedures for recovery of resistant starch;
f) method for the dissolution of RS; and
g) glucose determination procedure.

Incubations were performed at physiological temperature (37 °C) in both a shaking water bath and in an arrangement in which the contents of the tubes were continually stirred at different speeds. Incubations were allowed to proceed for up to 24 hours, and the RS values obtained for a set of samples were compared to values obtained from ileostomy studies. Typical results obtained for regular maize starch (RMS) and high-amylose maize starch (HAMS) are shown in Figure 7.1.

These incubations were performed in the presence of optimal levels of AMG and at a pH of 6.0. This pH was chosen as a compromise to allow for the different pH optima of α-amylase (pH 6.9) and AMG (pH 4.5) and the determined stability of the two enzymes on extended incubation at different pH values. At pH 6.0, pancreatic α-amylase has approximately 80% of the

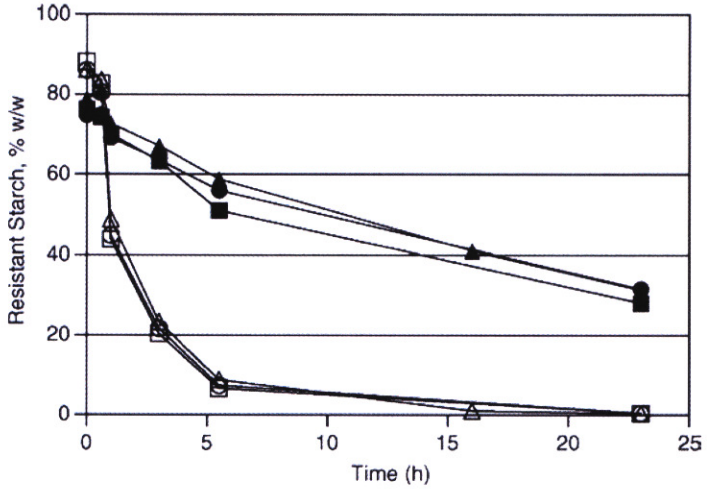

Figure 7.1 The effect of the concentration of pancreatic α-amylase and incubation time on the determined RS value of regular maize starch (RMS) and high-amylose maize starch (HAMS) at pH 6.0 and 37 °C for up to 25 hours. HAMS with pancreatic α-amylase concentrations of (black triangle) 15, (black circle) 30 and (black square) 60 U/mL; RMS with pancreatic α-amylase concentrations of (open triangle) 15, (open circle) 30 and (open square) 60 U/mL.

activity at the optimal pH of 6.9, and AMG has 20% of the activity at the optimal pH of 4.5. The level of each enzyme is adjusted to optimize the assay.

AMG is very stable at both pH 6.0 and 6.9, losing less than 5% of initial activity on incubation at 37 °C for 16 hours. Pancreatic α-amylase is less stable, however; even at pH 6.0, approximately 30% of activity remains on incubation of the enzyme under assay conditions of 16 hours at 37 °C (compared to 50% at pH 6.9) (Figure 7.2).

There was considerable flexibility in the concentration of pancreatic α-amylase used. RS values obtained for RMS and HAMS varied little, with enzyme concentrations ranging from 15–60 units/ml of incubation mixture (see Figure 7.1).

AMG in the incubation mixture had a considerable effect on the determined RS values (McCleary & Monaghan, 2002); this is considered to be due to the known inhibitory effect of maltose on pancreatic α-amylase. The AMG removed the maltose by hydrolyzing it to glucose, which has no inhibitory effect. The effect of protease on determined RS values was studied by including a pre-treatment with pepsin at pH 2. Results obtained indicated that the protease pre-treatment had no significant effect on determined RS values (McCleary & Monaghan, 2002). This may be due, in part, to the presence of an active protease in the pancreatic α-amylase preparation used.

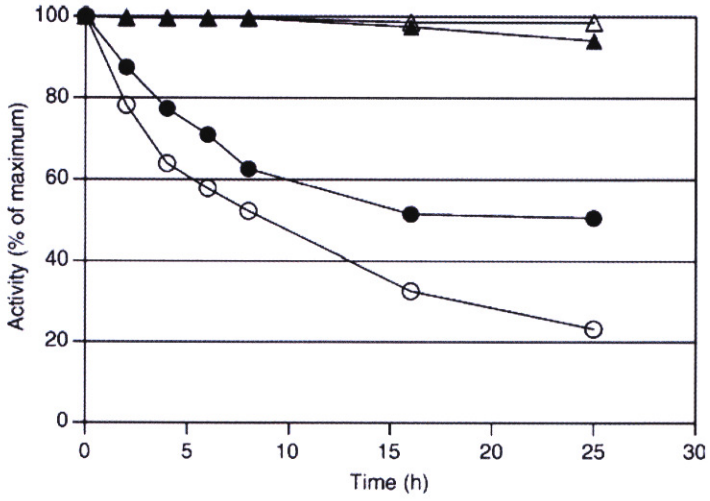

Figure 7.2 Stability of pancreatic α-amylase and AMG on incubation at pH 6.0 and 6.9 and 37 °C for up to 25 hours. AMG at pH 6.0 (closed triangle) and 6.9 (open triangle); pancreatic α-amylase at pH 6.0 (open circle) and 6.9 (closed circle).

Resistant starch is crystalline and is difficult to dissolve. Solvents used to dissolve this material include dimethyl sulphoxide (DMSO) and 2–4 M potassium hydroxide. For all samples studied, the RS was completely dissolved by stirring the RS containing pellet in 2 M KOH in an ice/water bath for 20 minutes (conditions used by several authors). On neutralization, it was essential to hydrolyze this starch rapidly to avoid re-crystallization (which would again render the starch resistant to hydrolysis by AMG. To achieve this and to simplify neutralization, a concentrated sodium acetate buffer (1.2 M, pH 3.8) was added, followed immediately by AMG (320 units/test; one unit of AMG activity is the amount of enzyme required to release one micromole of glucose from soluble starch per minute at pH 4.5 and 40 °C).

This method for the measurement of RS was subjected to evaluation under the auspices of AOAC International and AACC International, to determine the interlaboratory performance statistics. The materials used in the study represented food materials containing RS (cooked kidney beans, green banana and cornflakes) and a range of commercial starches, most of which naturally contain (or were processed to contain) elevated RS levels. Thirty-seven laboratories tested eight pairs of blind duplicate starch or plant material samples, with RS values between 0.6 (regular maize starch) and 64% (fresh weight basis). For samples excluding regular maize starch, RSD_r values

ranged from 1.97–4.2% and RSD_R values ranged from 4.58–10.9%. The range of applicability of the test is 2–64% RS.

The method was not suitable for samples with less than 1% RS (e.g. regular maize starch; 0.6% RS). For such samples, RSD_r and RSD_R values are unacceptably high. On the basis of this evaluation, the method was accepted as AOAC Official Method 2002.02 and AACC Recommended method 32–40.01 (McCleary *et al.*, 2002).

7.3 DEVELOPMENT OF AN INTEGRATED PROCEDURE FOR THE MEASUREMENT OF TOTAL DIETARY FIBRE

Hipsley (1953) coined the term *dietary fibre* to cover the non-digestible constituents of plants that make up the plant cell wall (known to include cellulose, hemicellulose and lignin) with the aim of defining some property of the constituent of the food that could be related to physiological behaviour in the human small intestine. This definition was broadened by Trowell *et al.* (1976) 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).

Methods which were developed to meet this analytical requirement focused on the use of enzymes to remove starch and protein. The enzymes employed require a defined level of activity and must be devoid of contaminating enzymes active on dietary fibre components such as pectin, β-glucan, arabinoxylan and other hemicelluloses. 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, 1994) This method was subsequently extended to allow measurement of total dietary fibre (TDF), soluble dietary fibre (SDF) and insoluble dietary fibre (IDF) in foods (AOAC Official Method 991.43) (Lee *et al.*, 1992) Other modifications to these methods for fibre analysis have also been approved by AOAC International (Theander & Aman, 1982).

In concurrent research in the UK, methods were developed for the measurement of non-starch polysaccharides (NSP)(Englyst *et al.*, 1982; Englyst & Cummings, 1984, 1985; Englyst & Hudson, 1987), based on the original work of Southgate (1969) and Southgate *et al.* (1978). These NSP procedures measure only NSP; RS and NDO are excluded. Starch in the sample is completely dissolved in hot dimethyl sulphoxide (DMSO), diluted in buffer and depolymerized with thermostable α-amylase, followed by a mixture of

pancreatin and pullulanase. The NSP recovered is acid hydrolyzed to monosaccharides, which are measured by high-performance liquid chromatography (HPLC), gas-liquid chromatography (after derivatization) (Englyst & Cummings, 1984) or colorimetrically (Englyst & Hudson, 1987). These methods have not been successfully subjected to international interlaboratory evaluation.

A survey of scientists initiated in 1993 (Lee & Prosky, 1995) showed that 65% of the respondents favoured the inclusion of NDO and 80% favoured inclusion of RS in the definition of dietary fibre. This led to the development of methods for measurement of RS (AOAC Method 2002.02) and for a number of NDO, including fructo-oligosaccharides (AOAC Methods 997.08 and 999.03), polydextrose (AOAC Method 2000.11), resistant maltodextrins (AOAC Method 2001.03), and galacto-oligosaccharides (AOAC Method 2001.02).

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 website, 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 (Anon, 2001) namely:

> *'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.'*

Several definitions of dietary fibre have appeared over the past ten years. The Food Nutrition Board of the Institute of Medicine of the National Academies (USA) (2002) defined dietary fibre as follows: '*Dietary fibre consists of nondigestible carbohydrates and lignin that are intrinsic and intact in plants. Added fibre consists of isolated, nondigestible carbohydrates that have beneficial physiological effects in humans. Total fibre is the sum of dietary fibre and added fibre.*'

At the 30th session of the CODEX Committee on Nutrition and Foods for Special Dietary Uses (CCNFSDU; 2008), the Committee agreed on the following definition for dietary fibre:

'Dietary fibre is carbohydrate polymers[1] *with ten or more monomeric units,*[2] *which are not hydrolyzed by the endogenous enzymes in the small intestine of humans and belong to the following categories:*

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

The fact that a single method to measure all dietary fibre components is needed has been known for some time. While it is possible to measure many individual fibre components with specific and non-specific methods, total dietary fibre cannot simply be calculated by adding the values for these specific components to the determined value of high molecular weight dietary fibre, as measured with AOAC Official Methods 985.29 or 991.43. Since these latter methods also measure some of the RS and various NDO in food materials, summation leads to 'double counting' of this material (Figure 7.3; McCleary *et al.*, 2009).

An integrated method for the measurement of total dietary fibre was published in 2007 (McCleary, 2007). This method allows the accurate measurement of total high molecular weight dietary fibre (HMWDF), which includes IDF (including RS) and higher molecular weight soluble dietary fibre which precipitates in the presence of 76% aqueous ethanol (SDFP), as well as lower molecular weight soluble dietary fibre which remains soluble in the presence of 76% aqueous ethanol (SDFS). Details of this procedure are outlined in Figure 7.4.

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[1] *When derived from a plant origin, dietary fibre may include fractions of lignin and/or other compounds when associated with polysaccharides in the plant cell walls and if these compounds are quantified by the AOAC gravimetric analytical method for dietary fibre analysis: Fractions of lignin and the other compounds (proteic fractions, phenolic compounds, waxes, saponins, phytates, cutin, phytosterols, etc.) intimately 'associated' with plant polysaccharides in the AOAC 991.43 method. These substances are included in the definition of fibre insofar as they are actually associated with the poly- or oligo-saccharidic fraction of fibre. However when extracted or even re-introduced in to a food containing non digestible polysaccharides, then they cannot be defined as dietary fibre. When combined with polysaccharides, these associated substances may provide additional beneficial effects.*

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

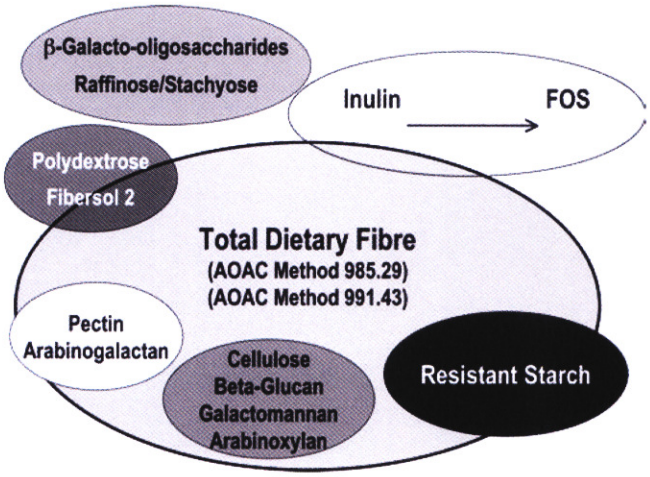

Figure 7.3 Schematic representation of dietary fibre components measured, and not measured, by AOAC Official Methods 985.29 and 991.43. Also depicted are the problems of partial measurement of RS, Polydextrose® and resistant maltodextrins by current AOAC total dietary fibre methods. Most of the SDFS (galactooligosaccharides, fructooligosaccharides, etc) are not measured. The currently described integrated total dietary fibre procedure measures all components shown, with no double counting.

The use of pancreatic α-amylase more closely simulates digestion in the human digestive tract and yields RS values in line with those obtained with AOAC Official Method 2002.02, and with results from ileostomy patients. For most food and ingredient samples analyzed, the RS values obtained with AOAC Method 2009.01 were higher than those obtained with AOAC Method 985.29. The notable exception is for phosphate cross-linked starch (RS4), where values obtained with Method 2009.01 are much lower than those obtained with Method 985.29. The physiological significance of these results will be discussed separately (McCleary *et al.*, 2013).

This method was successfully subjected to interlaboratory evaluation (McCleary *et al.*, 2009) and accepted as AOAC Method 2009.01. In this study, total HMWDF and SDFS were measured.

In an AOACI/AACCI interlaboratory study recently completed, the method was evaluated for the measurement of IDF, SDFP and SDFS. IDF and SDFP are measured using the standard gravimetric procedures, with allowance for ash and non-digested protein. SDFS is analyzed by HPLC using D-sorbitol as internal standard and a Waters Corporation Sugar Pak® chromatographic column. To obtain resistant starch values in line with those obtained *in vivo* with ileostomy patients, incubations are performed either in a shaking water

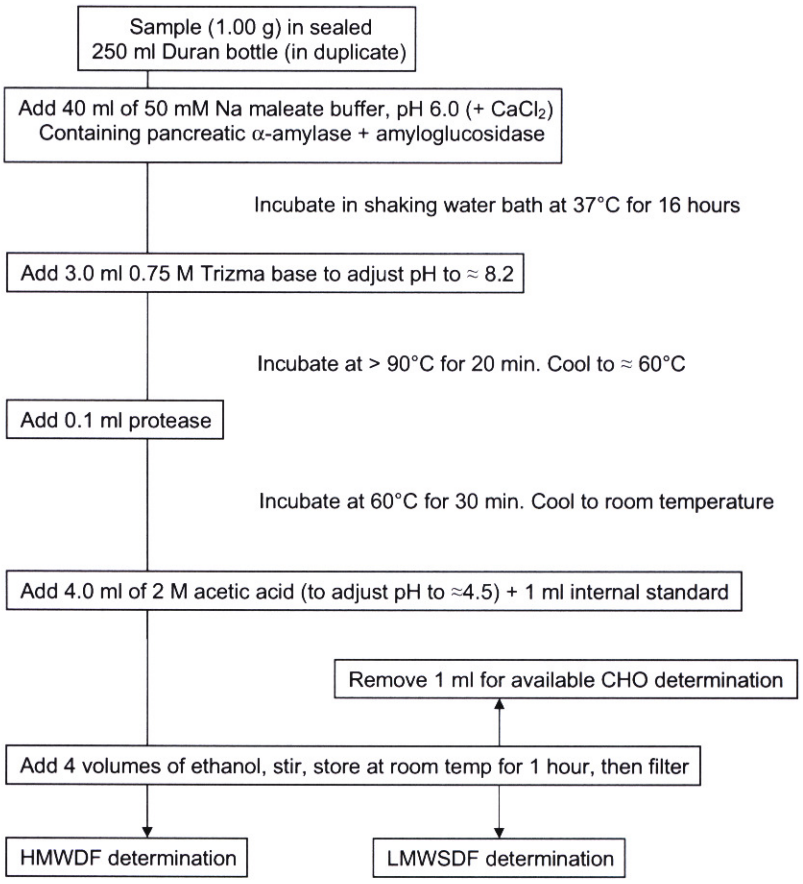

Figure 7.4 Schematic representation of the integrated TDF assay procedure, also showing where samples can be removed for determination of available carbohydrates.

bath in orbital motion, or with a VarioMag® magnetic stirrer at 37 °C for 16 hours. The pH is adjusted, and α-amylase and AMG are inactivated and protein denatured by heating the sample to about 100 °C. Incubation with protease is followed by pH adjustment and filtration to separate IDF and soluble fibres. SDFP is precipitated with ethanol and recovered and dried. After weighing, IDF and SDFP residues are analyzed for residual protein and ash. The SDFS fraction (in the ethanolic filtrate) is concentrated by rotary evaporation, re-dissolved and adjusted to pH 4.2–4.7 and incubated with AMG to remove completely any traces of higher molecular weight

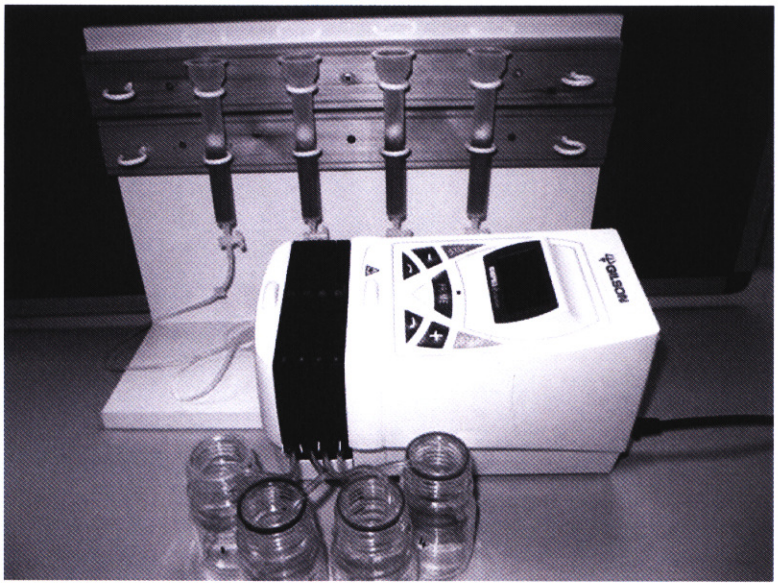

Figure 7.5 Arrangement for the deionization of samples using a mixed bed resin in a Bio-Rad, Econo-Pac® disposable chromatographic column (cat. No. 732–1010) with an Alltech One-Way Stopcock (cat. No. 211524). Also shown is a Gilson Minipuls® Evolution pump and collection bottles.

maltodextrins. The solution is then either desalted by passage through a column of mixed cation and anion ion exchange resins (Figure 7.5) or, alternatively, is desalted on line using a de-ashing pre-column.

Quantitation is greatly simplified by including an internal standard. The preferred internal standard is D-sorbitol and this is added to the sample just prior to adding ethanol to precipitate the SDFP. A number of compounds were evaluated as potential internal standards, including 1,5-pentanediol, diethylene glycol and triethylene glycol. Of these, diethylene glycol appeared best. However, on closer study, some of this is lost when the SDFS fraction is rotary evaporated, presumably by adsorbing to the glass rotary evaporator flask. This was not observed with D-sorbitol. When D-sorbitol is rotary evaporated with a range of sugars and NDO, the ratio of the components remains the same. Results of this interlaboratory study have been published (McCleary *et al.*, 2012).

A major advantage of the described method for the measurement of total dietary fibre is that it allows the separate measurement of IDF, SDFP and SDFS. There is some international debate as to whether NDO (SDFS) should be included in the dietary fibre measurements. Until there is agreement, this

oligosaccharide material can be measured and simply reported as NDO. With minor modification, the method can also be adapted to measure digestible carbohydrates (fructose, glucose, sucrose, maltodextrins and non-resistant starch, and the glucose component of lactose; McCleary, 2007).

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