8 Measurement of Dietary Fibre Components: the Importance of Enzyme Purity, Activity and Specificity

Barry V. McCleary

8.1 Total dietary fibre: introduction

Interest in dietary fibre is undergoing a dramatic revival, thanks in part to the introduction of new carbohydrates as dietary fibre components. Much emphasis is being placed on determining how much fibre is present in a food. Linking a particular amount of fibre to a specific health benefit is now an important area of research.

The term 'dietary fibre' first appeared in 1953, and referred to hemicelluloses, celluloses and lignin (Theander *et al.* 1995). Trowell (1974) recommended this term as a replacement for the no longer acceptable term 'crude fibre'. Burkitt (1995) has likened the interest in dietary fibre to the growth of a river from its first trickle to a mighty torrent. He observes that dietary fibre 'was first viewed as merely the less digestible constituent of food which exerts a laxative action by irritating the gut', thus acquiring the designation 'roughage' – a term later replaced by 'crude fibre' and ultimately by 'dietary fibre'. Various definitions of dietary fibre have appeared over the years, partly due to the various concepts used in deriving the term (i.e. origin of material, resistance to digestion, fermentation in the colon, etc.), and partly to the difficulties associated with its measurement and labelling (Mongeau *et al.* 1999). The principal components of dietary fibre, as traditionally understood, are non-starch polysaccharides (which in plant fibre are principally hemicelluloses and celluloses), and the non-carbohydrate phenolic components, cutin, suberin and waxes, with which they are associated in nature. In 1976, the definition of dietary fibre was modified to include gums and some pectic substances, based on the resistance to digestion of these components in the upper intestinal tract. For the purposes of labelling, Englyst *et al.* (1987) proposed that dietary fibre be defined as 'non-starch polysaccharides (NSP) in the diet that are not digested by the endogenous secretions of the human digestive tract'. Methods were concurrently developed to specifically measure NSP (Englyst *et al.* 1994).

8.1.1 *Measurement of NSP*

In the Englyst procedure (Englyst *et al.* 1994; Quigley and Englyst 1994) for the measurement of NSP, the sample is first defatted (if necessary) and then starch is completely removed. Starch removal is achieved by cooking the sample in dimethyl sulphoxide (which completely solubilises the starch), treatment with bacterial α-amylase and pullulanase, and finally precipitation of the dissolved fibre components with acidic alcohol. The starch fragments remain in solution and are removed. Pancreatin is added with the pullulanase to effect protein degradation. The precipitated material is recovered by centrifugation, washed with ethanol and acetone, and dried. This residue is acid hydrolysed and the sugar and uronic acid components are quantified by GLC, HPLC or by a colorimetric procedure. This procedure is used in the United Kingdom, but in other countries the AOAC dietary fibre procedures are the methods of

choice. The Englyst procedure has not received widespread adoption because, from a physiological point of view, NSP is less relevant than total dietary fibre. It is now generally accepted that resistant starch should be considered as part of dietary fibre.

8.1.2 *Measurement of total, soluble and insoluble dietary fibre*

An alternative – and now generally accepted – method for the measurement of dietary fibre is AOAC Method 985.29 (Prosky 1985), which has been modified to allow measurement of soluble and insoluble components (Prosky *et al.* 1988), and to allow the use of alternative buffers (AOAC method 991.43) (Lee *et al.* 1992). In principle, samples are treated with petroleum ether (if necessary) to remove fat, and then with enzymes to depolymerise starch and protein, which are subsequently removed in an ethanol precipitation step. The residue is dried and weighed and samples are taken for protein and ash determination. An extension of this method involves acid hydrolysis of the residue and analysis for sugars and uronic acids (the Uppsala method; AACC Method 32-25) (AACC 1997).

These methods are the culmination of the work of several research groups over many years. A major difference between these methods and the NSP procedure is that values obtained using the AOAC procedures include resistant starch and lignin. Resistant starch should be included under the 'umbrella' of dietary fibre, because it behaves in a manner similar to other dietary fibre components, in that it resists digestion in the small intestine and it is largely fermented in the colon, producing short-chain fatty acids (Champ *et al.* 1999).

8.1.3 *Enzyme activity*

Measurement of total dietary fibre (TDF), soluble dietary fibre (SDF) and insoluble dietary fibre (IDF) by AOAC methods (985.29, 991.42, 993.19) requires the use of three enzymes, thermostable α-amylase (*Bacillus licheniformis*), amyloglucosidase (*Aspergillus niger*) and protease (*Bacillus licheniformis*). The effectiveness and purity of these enzymes for dietary fibre analyses is usually determined by analysing specific control samples and monitoring recovery, e.g. starch, casein, β-glucan, pectin and larch galactan. With the first two samples, the recovery should be close to zero, whereas with the latter three, the recovery should be approximately 100%. The actual activity of the individual enzymes and of contaminants of importance is usually not provided or measured, and this is due (in part) to the absence of generally accepted assay methods. To fill this need, traditional methods for the measurement of each of these activities were evaluated, and new rapid and quantitative methods for the measurement of the major activities and important contaminants were developed (McCleary 1999).

Two methods were evaluated for the measurement of α-amylase in the commercially available thermostable α-amylase preparations used for dietary fibre analyses (McCleary 1999). One of these methods was based on the Nelson/Somogyi reducing sugar procedure (Somogyi 1952) with soluble starch as substrate, and the second used a defined *p*-nitrophenyl-maltosaccharide as substrate, in the presence of a thermostable α-glucosidase (Ceralpha method using Amylase HR reagent) (McCleary & Sheehan 1987) (Fig. 8.1). It was found that the activity of α-amylase preparations currently available from major suppliers is similar, i.e. ~10 000 U/ml (soluble starch) or ~3000 U/ml (Ceralpha method). However, the activity of preparations of this enzyme supplied for use in AOAC dietary fibre procedures over the past five years have ranged from 2000 to 10 000 U/ml (activity on soluble starch). From the results

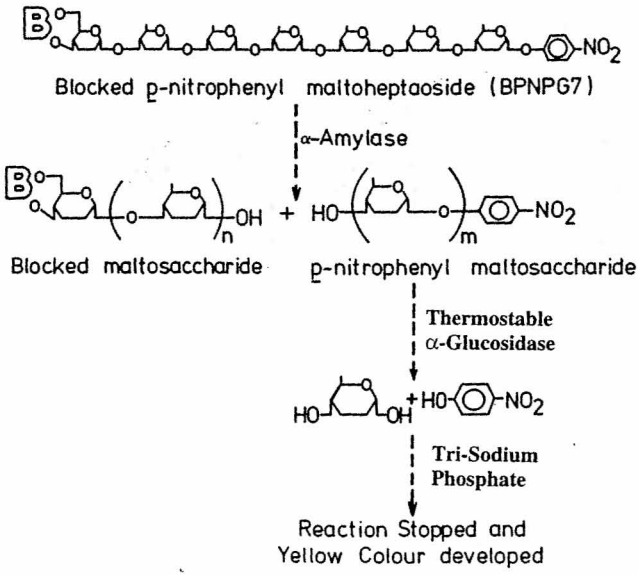

Fig. 8.1 Schematic representation of the measurement of α-amylase with Ceralpha reagent.

shown in Table 8.1, it is evident that the level of activity of this enzyme significantly affects the measured dietary fibre levels of certain samples, particularly resistant starch. Consequently, it is essential that the level of activity of this preparation be more clearly defined than has previously been the case. Since the preparations that are currently available commercially have an activity of approximately 10 000 ± 1000 U/ml on soluble starch (or 3000 ± 300 Ceralpha Units/ml), it would seem wise to set the required activity at this value.

Standardisation of the concentration of this enzyme is necessary to reduce the interlaboratory variations in reported values for resistant starch samples. From Table 8.1, it can be seen that for four commercial resistant starch materials (resistant starch A, B and C, and high-amylose maize starch), the level of α-amylase used in the assay has a very significant effect on the measured dietary fibre level. In AOAC Method 985.29, the recommended addition of α-amylase is 50 µl of preparations which are ~3000 Ceralpha Units/ml (i.e. 150 U/assay). It is evident, that higher or lower levels of enzyme will alter the determined dietary fibre levels for resistant starch samples. These variations have in the past caused conflict between analytical laboratories, but hopefully can now be avoided by using properly standardised enzyme preparations. Both described assays for the measurement of α-amylase give reliable and consistent results in the hands of a competent biochemist, but the Ceralpha method has the advantage of being extremely easy to use, even by analysts less experienced in biochemical assays.

Two methods have been compared for the assay of amyloglucosidase (McCleary 1999). One of these methods is more conventional; it employs soluble starch as substrate and the release of glucose is measured using glucose oxidase/peroxidase reagent. A second method employs *p*-nitrophenyl β-maltoside (PNPBM) as substrate in the presence of saturating levels of β-glucosidase (McCleary *et al.* 1991). In this method, as amyloglucosidase hydrolyses the terminal α-linked D-glucosyl residue, the excess levels of β-glucosidase give immediate

Table 8.1 Effect of the concentration of thermostable α-amylase and amyloglucosidase on determined dietary fibre contents of starch and resistant starch samples.

Polysaccharide	Enzyme preparation	Quantity (U/assay)	Total dietary fibre (%)
High-amylose* maize starch	Thermostable α-amylase‡	600 U	15.2
		300 U	23.4
		150 U	29.3
		75 U	34.2
		38 U	34.6
		19 U	38.3
Resistant starch A*	Thermostable α-amylase	600 U	29.5
		300 U	32.6
		150 U	34.8
		75 U	35.5
		38 U	35.8
		19 U	38.7
Resistant starch B*	Thermostable α-amylase	150 U	53.8
		75 U	55.5
		38 U	59.5
		19 U	61.0
Resistant starch C*	Thermostable α-amylase	600 U	28.1
		300 U	38.4
		150 U	43.3
Regular maize starch†	Amyloglucosidase§	40 U	0.08 ± 0.07
	Amyloglucosidase	20 U	1.3 ± 0.07
High-amylose maize starch*	Amyloglucosidase	120 U	28.8
	Amyloglucosidase	40 U	29.3
Casein†	Standard enzymes		1.38 ± 0.04
Pectin (ex. Sigma)†	Standard enzymes		89.0 ± 0.9
Pectin (ex. Megazyme)†	Standard enzymes		86.5 ± 1.0

* These samples were analysed once.

† Duplicate analyses were performed on these enzymes.

‡ α-Amylase activity is expressed as Ceralpha units. For all samples, an aliquot (50 µl) of enzyme is used, and the activities of the preparations are 380 to 12 000 U/ml. In the standard AOAC procedure, the concentration of the α-amylase is 3000 U/ml (or 150 U/assay).

§ Amyloglucosidase activity is expressed as PNP-Units bases on the assay using *p*-NP-β-maltoside.

hydrolysis of the β-linked D-glucosyl residue, releasing free *p*-nitrophenol that is detected by adding an alkaline solution. Both methods give quantitative measurement of amyloglucosidase. The level of activity of the amyloglucosidase preparation supplied by Megazyme is 200 U/ml on *p*-nitrophenyl β-maltoside (or 3300 U/ml on soluble starch), and 0.2 ml is used in the procedure (i.e. 40 U/test sample). Amyloglucosidase preparations supplied for use in TDF assay procedures have traditionally had an activity of about 130 U/ml on PNPBM (or ~2000 U/ml on soluble starch), and 0.3 ml of enzyme was used in standard AOAC methods (i.e. 40 U/test sample). This level of activity is adequate to ensure that all α-limit dextrins that are released on hydrolysis of starch by α-amylase, are hydrolysed to glucose in the standard assay format. The effect of using lower or higher levels of amyloglucosidase on the measured dietary fibre levels of regular maize starch and high amylose maize starch is shown in Table 8.1. With half of the recommended level of amyloglucosidase, the total dietary fibre values for regular maize starch increases only marginally (from <0.1% to 1.3%). Increasing the

level of amyloglucosidase to three times the recommended level has an insignificant effect on measured total dietary fibre in resistant starch samples, i.e. values for high-amylose maize starch decreased by less than 1%.

There are no reported problems associated with variations in the concentration of the protease used in dietary fibre determinations. This is most likely due to the fact that the level of enzyme used is more than adequate to give hydrolysis of all susceptible bonds in proteins in the samples being analysed. Also, any protein that is resistant to hydrolysis by the protease, and thus remains in the recovered fibre sample, is chemically measured and then subtracted in the calculations. However, since accurate and reliable assay procedures for standardising protease activity are desirable, two assay formats were evaluated (McCleary 1999). One method is a modification of traditional methods employing casein with TCA precipitation. This method was easy to use and give a linear standard curve over one absorbance unit. The second method employed Azo-Casein and a TCA precipitation step (Megazyme 1999). This assay has the advantage that it is more sensitive, the reaction products absorb in the visible range (440 nm) and the assay is specific for *endo*-protease. A standard curve for Subtilisin A on Azo-Casein is shown in Fig. 8.2. Activity Units for both assays were expressed as micromoles of tyrosine equivalents per min (Tyrosine Units). In evaluating enzyme preparations currently used in dietary fibre analyses (Subtilisin A), an activity of 6–8 tyrosine units/mg was obtained. In the standard AOAC dietary fibre methods, a protease concentration of 50 mg/ml is recommended (i.e. ~300–400 tyrosine Units/ml). Consequently, a concentration of 350 ± 50 U/ml was recommended for general use in dietary fibre determinations. A stabilised solution of this enzyme (350 U/ml) is commercially available (Megazyme cat. no. E-BSPRT).

8.1.4 *Enzyme purity*

Of the three enzymes used in dietary fibre determinations, only one – namely amyloglucosidase – was significantly contaminated with interfering activities. The thermostable

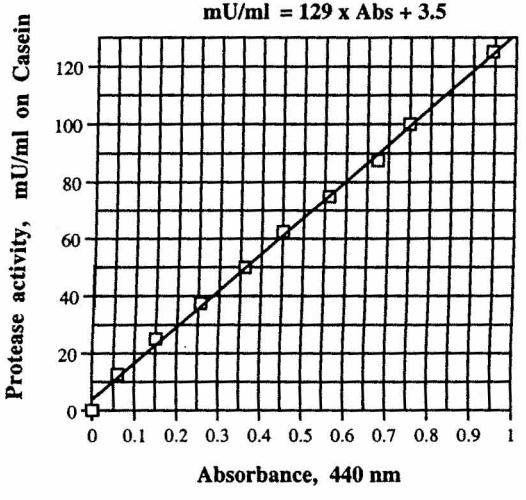

Fig. 8.2 Standard curve relating the activity of Subtilisin A on casein to action on Azo-casein (Megazyme Lot. 81001).

α-amylase and protease enzymes that are commercially available for use in dietary fibre measurement are generally free of interfering enzymes. Low levels of β-glucanase (*endo*-1,3:1,4-β–D glucanase; lichenase) have been detected in protease preparations, but these were well below the level which would interfere with the dietary fibre assay (i.e. that would result in a loss of β-glucan). The major contaminant in amyloglucosidase preparations (ex. *Aspergillus niger*) was shown to be *endo*-cellulase, and this resulted in *endo*-depolymerisation of mixed-linkage β-glucan from barley and oat, with a resultant underestimation of this component. The effect of cellulase contamination in amyloglucosidase preparations on the viscosity of barley β-glucan solutions is shown in Fig. 8.3, and the effect on analysed dietary fibre levels of pure barley β-glucan is shown in Table 8.2. It is evident that cellulase concentrations greater than 1.0 U/ml in the amyloglucosidase preparations (i.e. 0.2 U/assay) result in significant depolymerisation of barley β-glucan (Fig. 8.3) and loss of this component in dietary fibre analyses. Levels of cellulase equivalent to or higher than this concentration have been measured in some preparations of amyloglucosidase offered commercially for use in dietary fibre analyses. This contaminant can be readily detected and quantified using an assay procedure based on a substrate containing dyed, cross-linked β-glucan (Beta-Glucazyme tablets) (McCleary 1999). This simple test can assure that the enzyme is of adequate purity to ensure quantitative recovery of β-glucan, a major cereal based soluble dietary fibre component.

Another enzyme activity, which could cause significant underestimation of dietary fibre, particularly dietary fibre of fruit origin, is polygalacturonanase. This enzyme is most readily assayed using a viscometric method employing high-viscosity citrus pectin as substrate. It has been shown that the level of this enzyme in amyloglucosidase preparations used in

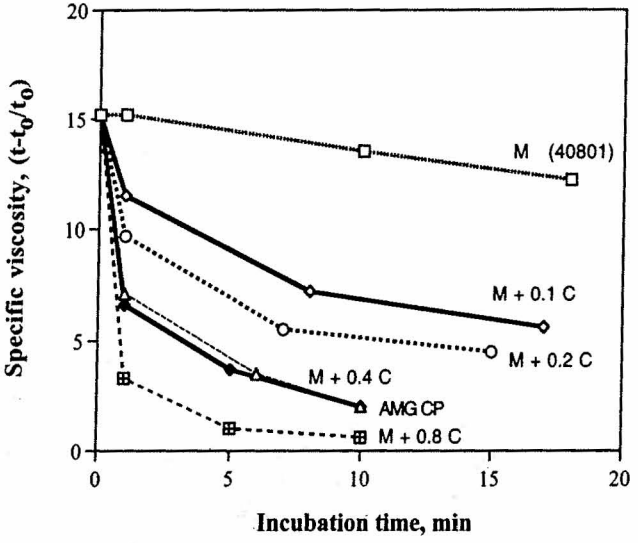

Fig. 8.3 Effect of *Aspergillus niger* cellulase on the viscosity of solutions of barley β-glucan. Assays were performed exactly as described in the text. To highly purified *A. niger* amyloglucosidase (lot 40801) cellulase was added at a concentration of 0.1, 0.2, 0.4 and 0.8 Units per 0.2 ml of amyloglucosidase, and 0.2 ml of these amyloglucosidase preparations were added to 10 ml of β-glucan solution (10 mg/ml, pH 4.5) in the viscometer.

Table 8.2 Effect of the concentration of cellulase contamination in amyloglucosidase on determined dietary fibre contents of pure barley β-glucan.

Polysaccharide	Added cellulase (Units/assay i.e./0.2 ml AMG)	Total dietary fibre (%)
Barley β-glucan (100 mg)*	0.0	99.9
	0.05	99.2
	0.10	99.1
	0.20	98.4
	0.40	90.0
	0.80	69.2

*All values are based on single determinations.

dietary fibre determinations were extremely low (McCleary 1999). On incubation of 0.2 ml of amyloglucosidase preparation (as used in dietary fibre analyses) with a solution of citrus pectin (10 ml, 10 mg/ml), there was only minor viscosity reduction over an incubation period of 20 min. This viscosity reduction was equivalent to a polygalacturonanase concentration of less than 0.01 U/assay (i.e. per 0.2 ml of amyloglucosidase), which is below the level which would result in pectin loss in AOAC dietary fibre assays (about 0.20 U/test). However, other enzyme preparations, that may be incorporated into AOAC total dietary fibre procedures, may contain this contaminating activity (e.g. Fructozyme® which is used to remove fructans) (Quemener *et al.* 1993, 1997).

The TDF values obtained for casein and pectin (ex. Megazyme TDF Controls Kit) and pectin from Sigma Chemical Co. (dietary fibre control) are also shown in Table 8.1.

The dietary fibre value obtained for casein was as expected, but the value for the control pectin samples was much lower than expected. This was not due to a pectinase contamination in the amyloglucosidase, as the level of this activity in the enzyme used (amyloglucosidase, E-AMGDF, Megazyme International) was negligible (less than 0.01 U per 0.2 ml of amyloglucosidase) and the levels in protease and thermostable α-amylase preparations were undetectable. The low recovery must be due to incomplete precipitation of the pectin in 78% ethanol. The reason for this is not apparent, as both pectins used were quite pure, high-molecular weight materials.

8.2 Specific dietary fibre components

8.2.1 *1,3:1,4 β-D-Glucan (mixed-linkage β-glucan)*

Plant-derived dietary fibre is generally considered to include all plant components except protein, non-resistant starch, lipids and low-molecular weight materials not precipitated by 80% ethanol. The first of this mixture of components to attract individual attention was mixed-linkage β-glucan (β-glucan) from cereal grain sources, particularly barley and oats. The interest in this polysaccharide was catalysed by the numerous health claims concerning the use in human nutrition of oat fibre products rich in β-glucan (Anderson and Bridges 1993). The Food and Drug Administration (USA) has allowed the claim that the inclusion of oat products in the diet may reduce the risk of heart disease.

A specific procedure for the measurement of β-glucan was developed in 1985 (McCleary & Glennie-Holmes) (Fig. 8.4), and then further simplified (McCleary & Codd 1991). In prin-

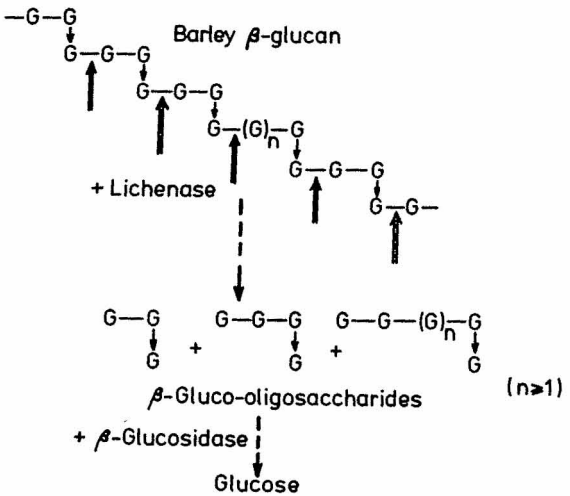

Fig. 8.4 Schematic representation of the measurement of β-glucan with lichenase and β-glucosidase (AOAC Method 995.16).

ciple, β-glucan in the sample is hydrated by cooking a slurry of the flour, followed by depolymerisation with a specific 1,3:1,4 β-glucanase (lichenase) enzyme. This enzyme cleaves the (1→4)-glycosidic linkage of a 3-linked D-glucosyl residue within the β-glucan chain and thus has no action on (1→4) β-linked D-glucans (cellulose). The oligosaccharide reaction products released on hydrolysis of β-glucan by lichenase are then hydrolysed to glucose with a highly purified β-glucosidase, and the glucose is measured enzymatically with glucose oxidase/peroxidase reagent. Since the procedure is used to measure trace levels of β-glucan in cereal and food products, which also contain high levels of starch and other glucose containing oligosaccharides, such as malto-oligosaccharides and sucrose, the enzymes must be very pure. Invertase contamination of β-glucosidase will give glucose release from sucrose, and contamination by α-glucosidase or amyloglucosidase will result in release of glucose from malto-oligosaccharides. In both cases, an inflated estimate of β-glucan content will result. The purity of β-glucosidase can be tested by incubating a range of concentrations of the enzyme with aliquots of lichenase treated barley flour slurry (as per the standard β-glucan assay format). If the enzyme is devoid of interfering activities, the same glucose absorbance value should be obtained. The results of such an experiment are shown in Fig. 8.5. Normally, 0.2 U of β-glucosidase is used per assay, but it is evident that even with a 75-fold excess (15 U/assay) of this enzyme, the determined β-glucan content is only very slightly (~3%) overestimated.

β-Glucosidase from *A. niger* is very effective in catalysing complete hydrolysis, to glucose, of the mixed-linkage β-gluco-oligosaccharides released on hydrolysis of barley or oat β-glucan by lichenase. However, this is not the case for the β-glucosidase from almond emulsin (Fig. 8.5). The almond enzyme, even at very high levels of activity, does not completely hydrolyse these oligosaccharides, leading to an underestimation of the β-glucan content of the sample.

Following extensive international interlaboratory evaluations, this assay procedure has become the International standard method for the measurement of mixed-linkage β-glucan

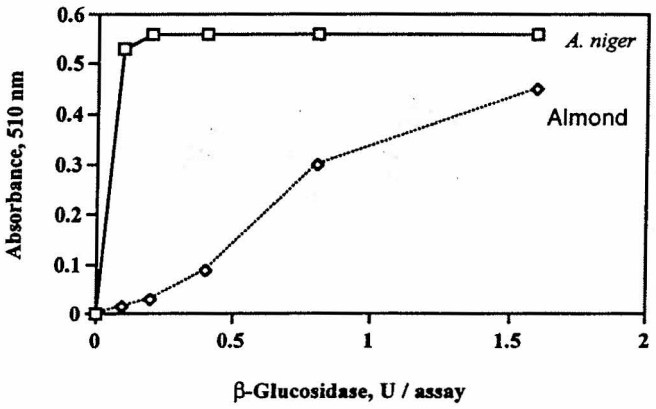

Fig. 8.5 Hydrolysis of β-gluco-oligosaccharides (from lichenase treatment of mixed-linkage β-glucan) by highly purified *A. niger* and almond-seed β-glucosidases.

(AOAC 995.16; AACC Method 32-23; ICC Standard No. 166; EBC Methods 3.11.1, 4.16.1 and 8.11.1). The method has been adapted for the analysis of mixed-linkage β-glucan in cereal and food products and in liquid materials such as wort and beer.

8.2.2 *Resistant starch*

Until recently, it was generally thought that starch was completely digested in the small intestine. However, it is now recognised that there is a portion (resistant starch, RS) which resists digestion, passes into the large intestine and is fermented there. Three types of RS have been identified: (1) physically trapped starch; (2) resistant starch granules; and (3) retrograded starch (Muir *et al.* 1993; Englyst *et al.* 1994). Physically trapped starch granules are locked within the food matrix, such that digestive enzymes are prevented or delayed from having access to them. Resistant starch granules, as are present in raw potatoes and green bananas, resist attack by α-amylase, which is probably due to the crystalline nature of the starch. From an industrial and food technology perspective, the most interesting resistant starches are those which are produced through retrogradation of gelatinised starches. Retrogradation is generally attributed to the amylose portion of the starch, so starches high in amylose are particularly amenable to the production of resistant starch.

Since resistant starch is defined as 'the sum of starch and products of starch degradation not absorbed in the small intestine of healthy individuals' (Asp 1992; Muir *et al.* 1993), any analytical method for RS should take into account all the starch and α-dextrins covered in this physiological definition. Furthermore, the method should be validated using *in-vivo* data from healthy human subjects. The various procedures for the measurement of resistant starch have been summarised and discussed by Champ *et al.* (1999). The methods can be broadly grouped into two approaches. In one approach (Englyst *et al.* 1992; Muir *et al.* 1993), samples are analysed for total starch and for soluble starch, and the latter is subtracted from the former to give resistant starch. The major inherent problem with this approach, analytically, is that the content of resistant starch is generally a small proportion of total starch. Thus, the resistant starch value is obtained by subtracting one large analytical value (for non-resistant starch) from another similar large value (for total starch), meaning that analytical precision is greatly

reduced. In the second approach (Champ 1992; McCleary *et al.* 1997), the sample is treated with starch-degrading enzymes to remove the non-resistant starch, which is washed away from the residue. The residue is then analysed. By definition, this approach must be analytically more precise. The procedure should be used in conjunction with AOAC Method 985.29 to determine total dietary fibre in the sample.

In most TDF procedures, the hydrolytic conditions are quite severe, resulting in underestimation of resistant starch. If the final total dietary fibre value is to be the sum of TDF determined by AOAC Method 985.29 plus resistant starch determined by a method such as that of Champ (Champ 1992; Champ *et al.* 1999), then the amount of resistant starch measured in AOAC Method 985.29, must be allowed for or removed (otherwise it will be counted twice). A possible way to handle this is to pre-treat samples with hot dimethyl sulphoxide (DMSO) or sodium hydroxide prior to TDF analysis by AOAC Method 985.29. The DMSO treatment should dissolve all of the starch, allowing complete hydrolysis by α-amylase and amyloglucosidase, and subsequent removal in the alcohol treatment step. Of course, such a treatment will alter the ratio of soluble to insoluble fibre as determined by AOAC Method 991.43. A DMSO treatment step is used in AOAC Method 996.11 for the measurement of total starch (McCleary *et al.* 1997) and in the Englyst *et al.* (1994) NDF procedure.

8.2.3 *Oligofructan and inulin*

Fructans are widely distributed in the plant kingdom. They are defined as any compound in which one or more fructosyl–fructose linkages constitute a majority of the linkages. They are generally classified as inulin, levan or graminan types, based on linkage types. In nature, inulin occurs as a series of oligosaccharides with degrees of polymerisation from 2 to 60 (Pontis 1990; Lewis 1993). It consists principally of linear chains of fructosyl units linked α-(2-1), ending with a glucosyl unit. Inulin and oligofructose resist hydrolysis by human digestive enzymes, but are fermented in the large intestine. They thus have the same physiological action as dietary fibre.

In recent years, interest in the measurement of fructans such as inulin and oligofructose has been stimulated by applications made to regulatory authorities for acceptance of fructans as dietary fibre components for food labelling purposes (Coussement 1999).

Since fructans are largely soluble in 80% ethanol, they are not significantly measured in currently used dietary fibre methods. Several methods have now been developed for the specific measurement of fructans, and these rely on the complete hydrolysis of the oligofructose and inulin to fructose and glucose, which are measured either instrumentally (Quemener *et al.* 1993; Hoebregs 1997) or chemically (McCleary & Blakeney 1999; McCleary *et al.* 2000). In some procedures, the separate quantities of fructan, sucrose, glucose and starch are determined by hydrolysis with specific enzymes, followed by chromatographic procedures (AOAC Method 997.08) (Hoebregs 1997). In a procedure developed in the author's laboratory (McCleary & Blakeney 1999), fructan is measured chemically, after removal of sucrose and starch through enzymatic hydrolysis, followed by borohydride reduction (Fig. 8.6). Sucrose is completely hydrolysed to glucose and fructose with a specific sucrose-degrading enzyme (sucrase; α-glucosidase) with no detectable hydrolysis of fructo-oligosaccharides, not even kestose (Fig. 8.7). The relative rates of hydrolysis of sucrose and kestose (under optimal assay conditions (i.e. pH 6.5 and 40°C, and at a substrate concentration of 10 mg/ml) was 3800 : 1. Since starch and maltosaccharides are unstable in the highly alkaline conditions used in the PAHBAH reducing sugar method, it is essential to remove these from the sample

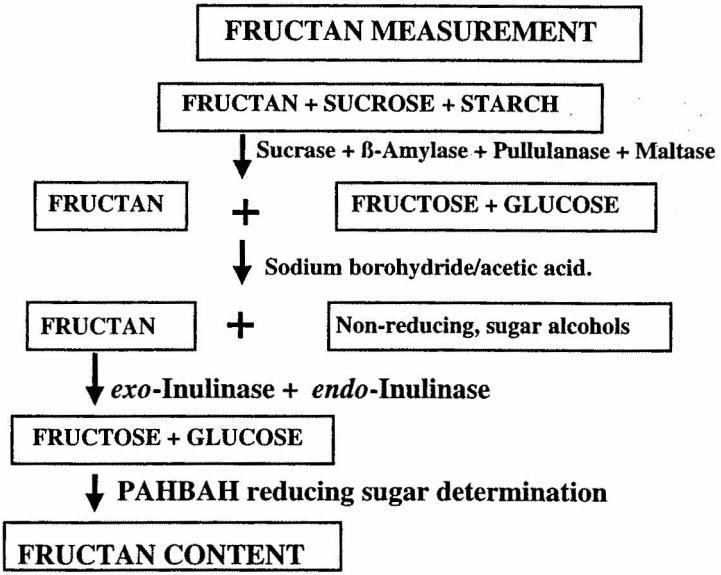

Fig. 8.6 Schematic representation of an enzymic procedure for the measurement of fructan (inulin) (AOAC Method 999.03).

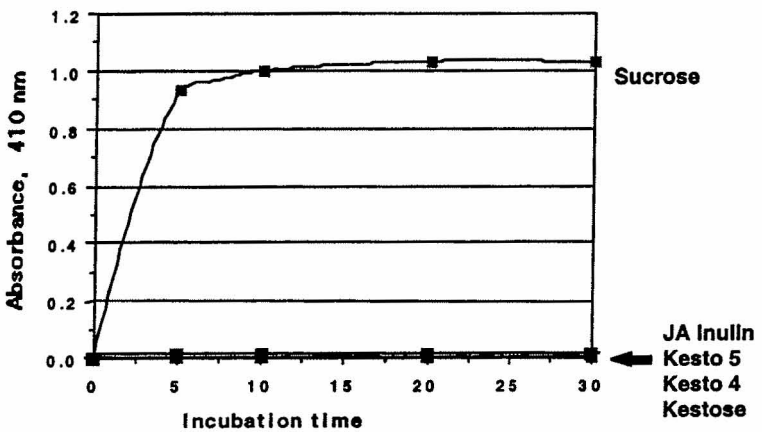

Fig. 8.7 Hydrolysis of sucrose and fructans by sucrase (α-glucosidase). Sugar compound (0.2 ml, 50 µg) was incubated with sucrase (1 unit) in sodium maleate buffer (100 mM, pH 6.5) at 40°C. The reaction was terminated at various time intervals with PAHBAH working reagent, and the colour developed.

extract. This is achieved by hydrolysis to glucose by the combined action of pullulanase, β-amylase and maltase, followed by borohydride reduction. The level of each enzyme used was 10 times that required for complete hydrolysis. This combination of enzymes was chosen to allow starch hydrolysis to be performed concurrently with hydrolysis of sucrose by sucrase (i.e. at pH 6.5 and 40°C).

In the development of the current procedure for the assay of inulin and fructo-oligosaccharides, two inulin-degrading enzymes were purified, *exo*-inulinase and *endo*-inulinase. Thin-layer chromatographic patterns of the products released on hydrolysis of onion, wheat and high degree of polymerisation (DP) chicory fructans by *exo*-inulinase showed that the only reaction products are fructose and glucose (McCleary 1999). Wheat and chicory fructan are hydrolysed less rapidly than onion fructan. The slower rate of hydrolysis of the wheat fructan is probably due to the highly branched nature of this mixture of oligosaccharides. The Bio-Gel P-2 chromatographic patterns of onion fructo-oligosaccharide mixture, before and after incubation with *exo*-inulinase (Fig. 8.8) demonstrate that with the level of enzyme used, hydrolysis to fructose and glucose is complete. Incubation of chicory fructan with *endo*-inulinase produces an accumulation of oligosaccharides of DP 4, 5 and 6.

In the final assay format, the inulin and oligofructose are hydrolysed to fructose and glucose using a highly purified *exo*-inulinase enzyme (to which a small amount of *endo*-inulinase has been added). The released sugars are measured with a reducing-sugar method. In initial work, it was considered that rapid hydrolysis of high-molecular weight inulin by *exo*-inulinase may require the presence of *endo*-inulinase. However, as shown in Table 8.3, addition of *endo*-inulinase (at 10% the level of *exo*-inulinase) has an insignificant effect on the level of measured inulin, when more than 18 U of *exo*-inulinase is used per assay. A major problem with the addition of *endo*-inulinase is the fact that in the purification of this enzyme it is difficult to completely remove β-glucosidase and cellulase. After extensive purification, contamination of *endo*-inulinase with β-glucosidase is about 1%, and with cellulase is 0.001%. When this is mixed with *exo*-inulinase at a ratio of 1 : 10, the final contamination with β-glucosidase is thus just 0.1%. However, this level of contamination is sufficient to give an overestimation of the fructan content of cereal flours (up to 20%) through hydrolysis of β-glucan and release of glucose. For this reason, the level of *endo*-inulinase in the fructanase mixture used in the standard fructan assay procedure has been reduced to just 1% of the *exo*-inulinase. Under these conditions, the level of contaminating β-glucosidase has an insignificant effect.

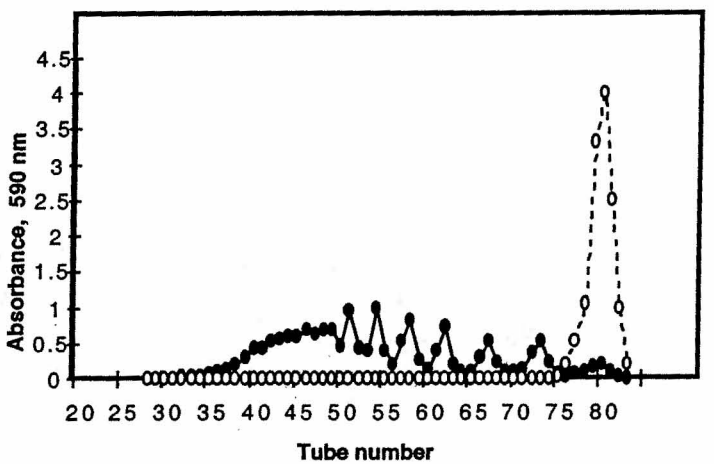

Fig. 8.8 Bio-Gel P-2 chromatography of the sugars produced on hydrolysis of onion fructan by *exo*-inulinase. Column eluates were analysed by the phenol–sulphuric acid procedure. Incubated for 0 min (•) and 60 min (o).

Table 8.3 Effect of concentration of *exo*-inulinase and presence or absence of *endo*-inulinase on the determined fructan content of selected samples.

Sample and amount (U/assay) of exo-inulinase*	Determined fructan content, % w/w (db)	
	exo-inulinase	*exo*- plus *endo*-inulinase
Dehydrated onion		
36	58.8	61.1
18	58.6	58.9
9	57.8	58.0
4.5	57.2	55.6
Wheat stalks		
36	5.3	5.3
18	5.1	5.1
9	4.7	4.6
4.5	4.2	4.2
Chicory		
36	98.7	98.9
18	93.4	97.8
9	90.0	93.4
4.5	81.8	83.5

* The level of *endo*-inulinase in each incubation was 10% of the level of *exo*-inulinase.

exo-Inulinase acts on galactosyl-sucrose oligosaccharides (e.g. raffinose and sucrose) to produce a series of galactosyl-glucose oligosaccharides (e.g. melibiose from raffinose). Thus, the presence of these oligosaccharides in the samples that are being analysed will result in an overestimation of the fructan content. This may be of significance in the analysis of food materials that contain legume seed material as well as fructo-oligosaccharides. With such samples, if an accurate measurement of fructo-oligosaccharides is required, then extracts of the samples being analysed should be pre-treated with α-galactosidase (plus sucrase) to catalyse complete hydrolysis of galactosyl-sucrose oligosaccharides to galactose, glucose and fructose. This is best performed in the pre-incubation step in which sucrose and starch are hydrolysed and removed (by borohydride reduction).

The specific advantages of this assay procedure for inulin and fructo-oligosaccharides are that highly purified enzymes are used and the assay can be performed with basic laboratory equipment. The method has been evaluated on a range of fructan-containing materials, including both natural and compounded food products, and has been subjected to an extensive international interlaboratory evaluation under the auspices of AOAC International (McCleary *et al.* 2000). This study involved 15 laboratories and 18 samples (nine blind duplicates). Samples included a range of native materials as well as a range of commercial food products such as milk powder, chocolate and low-fat spreads (to which inulin had been added). Repeatability relative standard deviations (RSD_r) ranged from 2.3 to 7.3% and reproducibility relative standard deviations (RSD_R) ranged from 5.6 to 11.5%. The mean value obtained for 'pure' fructan was 95.8% (on an as-is basis. On the basis of these results, the method has been recommended by AOAC International for First Action Approval (Method 999.03; McCleary *et al.* 2000).

Fructans comprise a series of oligosaccharides, some of which are precipitated with alcohol in the total dietary fibre assay procedure, but others – the lower DP oligomers – remain

soluble at this concentration of alcohol. This introduces a complication into dietary fibre analyses. A proposed solution to this problem (Quemener *et al.* 1993, 1997) is to treat samples for dietary fibre analysis with a fructanase mixture to catalyse completely depolymerisation of the fructan (and thus complete solubilisation in 80% alcohol). This step ensures that none of the fructan is measured in AOAC total dietary fibre procedure. The fructan is then measured separately and added to the dietary fibre value. For this modification to give reliable results, the fructanase mixture used to hydrolyse the fructan must be devoid of enzymes active on other dietary fibre components. Quemener *et al.* (1997) recommended the use of heat-treated Fructozyme®. Heat treatment was shown to effectively inactivate most of the pectin degrading activities in the preparation, and resulted in quantitative pectin measurement in the AOAC total dietary fibre method 985.29. However, Fructozyme® also contains high levels of α-galactosidase and β-glucanase (cellulase) (Table 8.4). The β-glucanase is not inactivated by the heat treatment step recommended by Quemener *et al.* (1993) (60°C, 2 h) and gives a rapid depolymerisation of β-glucan, resulting in a significant underestimation of this component in AOAC method 985.29 (McCleary 1999). This β-glucanase contamination can, however, be removed chromatographically (Megazyme Data Sheet; E-FRMXLQ) (Table 8.5) allowing the use of the enzyme as proposed by Quemener *et al.* (1997).

8.2.4 *Polydextrose*

Polydextrose is prepared by vacuum thermal polymerisation of glucose, using sorbitol as plasticiser and citric acid as catalyst (Craig *et al.* 1999). This random polymerisation and branching yields various types of glucosidic bonds in the structure with α-1,6 bonds predominating. The compound includes covalent linkages to sorbitol and citric acid, and has an average degree of polymerisation of 12. Polydextrose is an approved food additive in over 40 countries. It is not hydrolysed by normal human digestive enzymes, but it is fermented in the colon like many other fibres.

Polydextrose is not quantitatively measured with AOAC Method 985.29 because, as is the case with other resistant oligosaccharides (RO), it is not completely precipitated with 80% ethanol. Because of the complex chemical nature of the compound, measurement cannot be achieved through specific enzymatic hydrolysis. Consequently, analytical procedures

Table 8.4 Levels of inulinases, cellulase, polygalacturonanase (pectinase) and α-galactosidase in Fructozyme®, heat-treated Fructozyme® and a purified fructanase mixture (ex. Megazyme).

	Enzyme activity (Units/ml)*		
	Fructozyme	HT Fructozyme†	Pure Fructanase Mixture‡
exo-Inulinase	2000*	1200	2000
endo-Inulinase	200	200	200
Pectinase	40	4.1	0.4
Cellulase	4.0	4.0	0.1
α-Galactosidase	284	10.2	0.05

* One Unit of activity is the amount of enzyme required to release 1 μmol product/min under standard assay conditions (40°C, pH 4.5).

† The Fructozyme was pre-heated (HT Fructozyme) at 60°C for 120 min.

‡ This preparation is available from Megazyme (cat. no. E-FRMXLQ).

Table 8.5 Effect of addition of crude and purified fructanases on the recovery of inulin and β-glucan in the AOAC International total dietary fibre assay procedure.

		Recovery (%)	
Enzyme preparation	Quantity	Inulin (Fructan)	β-Glucan
Heat-treated Fructozyme*	0.2 ml	0.2±0.2	11.4±0.4
	0.1 ml	0.2±0.2	52.0±2.0
	0.05 ml	7.2±0.3	80.0±1.2
Pure Fructanase mixture	0.2 ml	0.2±0.2	99.0±1.3
	0.1 ml	0.2±0.2	100.8±0.8
	0.05 ml	3.5±0.2	98.5±1.8

* All samples were analysed in duplicate.
The levels of enzyme activities in heat-treated Fructozyme and the pure fructanase mixture are as shown in Table 8.4. The amount of β-glucan used in assays was 100 mg.

depend on aqueous extraction of the sample being analysed, followed by enzymatic hydrolysis of other oligosaccharide and polysaccharide materials that are likely to interfere in subsequent chromatographic quantitation. Highly purified enzymes active on starch, cellulose, pectins and gums (e.g. galactomannans), have been employed (Craig *et al.* 1999).

8.2.5 *Galacto-oligosaccharides*

Galacto-oligosaccharides [α-D-Glc*p*(1–1)-β-D-Gal*p*-containing oligosaccharides) have been shown to be beneficial to human health through promotion of the growth of bifidobacteria in the large intestine (Fransen *et al.* 1998). The oligogalactosylated glucoses are prepared from lactose through the transgalactosylating activity of β-galactosidase. Various parameters, such as the source of the enzyme, substrate concentration, pH and temperature influence the transgalactosylation and thus, the final yields of the different products. Typically, the reaction products are composed of about 60% transgalacto-oligosaccharides (mainly tri-, tetra- and pentasaccharides) and about 40% of a mixture of lactose, glucose and galactose.

Alternative galacto-oligosaccharides are the galactosyl-sucrose oligosaccharides of plant origin, namely raffinose, stachyose and verbascose (Dey 1978). These oligosaccharides consist of sucrose, to which an -extended chain of 1,6 α-linked galactosyl residues is attached to C-6 of the glucosyl residue. Raffinose is a major by-product of sugar crystallisation from sugarbeet extracts, and it has been shown to promote the proliferation of bifidobacteria in the human intestinal microflora.

Acceptance of galacto-oligosaccharides as soluble dietary fibre components requires the development of analytical procedures that will specifically measure these components in food mixtures. The lactose-derived β-galacto-oligosaccharides are effectively hydrolysed with *A. niger* β-galactosidase, while the galactosyl-sucrose oligosaccharides are rapidly and quantitatively hydrolysed by *A. niger* α-galactosidase. The released sugars can be measured, either chromatographically, or using various colorimetric procedures. In both cases, the specificity of the procedure depends on the purity of the enzyme used. A procedure for the measurement of transgalacto-oligosaccharides is the subject of an upcoming AOAC International, interlaboratory study. A major consideration is the measurement of the transgalacto-oligosaccharides independently of lactose present in the sample.

8.3 Conclusions

In conclusion, success in the introduction of 'new' dietary fibre components will be dependent on identifying a specific health benefit of the component, as well as providing an analytical procedure that will allow accurate measurement of this component in complex food mixtures. Development of such procedures will require the use of highly purified enzymes of known activity and specificity to either hydrolyse the fibre component to components that can be analysed, or alternatively, to remove other fibre components which will interfere in the assay.

References

AACC (1997) American Association of Cereal Chemists. Total dietary fibre. Determination as neutral sugar residues, uronic acid residues and Klason lignin (Uppsala method). 9th Edition. Method 32-25. First approval 10-26-94. American Association of Cereal Chemists, St. Paul, MN.

Anderson, J.W. & Bridges, S.R. (1993) In: *Oat Bran.* (ed. P.J. Wood). American Association of Cereal Chemists Inc., pp. 139–157. St. Paul, MN, USA.

AOAC Official Method 985.29. (1995) Total dietary fiber in foods-enzymatic gravimetric method. In: *Official Methods of Analysis, 16th ed.* AOAC International, Arlington.

Asp, N.-G. (1992) Resistant starch. Proceedings from the 2nd plenary meeting of EURESTA – European Flair Concerted action on physiological implications of the consumption of resistant starch in man. *European Journal of Clinical Nutrition,* **46** (S2), S1.

Burkitt, D. (1995) Historical aspects. In: *Dietary Fibre in Health and Disease.* (eds D. Kritchevsky & C. Bonfield), pp. 3–10. Eagan Press, Minnesota.

Champ, M., Amado, R., Vanamelsvoort, J.M.M., *et al.* (1992) Determination of resistant starch in foods and food products – interlaboratory study. *European Journal of Clinical Nutrition,* **46** (S2), S51–S62.

Champ, M., Martin, L., Noah, L. & Gratas, M. (1999) Analytical methods for resistant starch. In: *Complex Carbohydrates in Food.* (eds S.S. Cho, L. Prosky & M. Dreher), pp. 169–187. Marcel Dekker Inc., New York.

Coussement, P. (1999) Inulin and oligofructose as dietary fibre: analytical, nutritional and legal aspects. In: *Complex Carbohydrates in Food.* (eds S.S. Cho, L. Prosky & M. Dreher), pp. 203–212. Marcel Dekker Inc., New York.

Craig, S.A.G., Holden, J.F., Troup, M.H. Auerbach, M.H. & Frier, H. (1999) Inulin and oligofructose as dietary fibre: analytical, nutrtional and legal aspects. In: *Complex Carbohydrates in Food.* (eds S.S. Cho, L. Prosky & M. Dreher), pp. 229–247. Marcel Dekker Inc., New York.

Dey, P.M. (1978) Biochemistry of plant galactomannans. *Advances in Carbohydrate Chemistry and Biochemistry,* **35**, 341–376.

Englyst, H.N., Trowell, H., Southgate, D.A.T. & Cummings, J.H. (1987) Dietary fibre and resistant starch. *American Journal of Clinical Nutrition,* **46**, 873–874.

Englyst, H.N., Kingman, S.M. & Cummings, J.H. (1992) Classification and measurement of nutritionally important starch fractions. *European Journal of Clinical Nutrition,* **46** (Suppl. 2), S33–S39.

Englyst, H.N., Quigley, M.E. & Hudson, G.J. (1994) Determination of dietary fibre as non-starch polysaccharides with gas-liquid chromatographic, high-performance liquid chromatographic or spectrophotometric measurement of constituent sugars. *Analyst,* **119**, 1497–1509.

Fransen, C.T.M, Van Laere, K.M.J, van Wijk, A.A.C., *et al.* (1998) α-D-Glc*p*-(1–1)-β-D-Gal*p*-containing oligosaccharides, novel products from lactose by the action of β-galactosidase. *Carbohydrate Research,* **314**, 101–114.

Hoebregs, H. (1997) Fructans in food and food products, ion-exchange-chromatographic method: collaborative study. *Journal of the Association of Official Analytical Chemists,* **80**, 1029–1037.

Lee, S.C., Prosky, L. & DeVries, J.W. (1992) Determination of total, soluble and insoluble dietary fibre in foods – enzymic gravimetric method, MES-Tris buffer: collaborative study. *Journal of the Association of Official Analytical Chemists,* **75**, 395–416.

Lewis, D.H. (1993) Nomenclature and diagrammatic representation of oligomeric fructans – a paper for discussion. *New Phytologist* **124**, 583–594.

McCleary, B.V. (1999) Enzyme purity and activity in fibre determinations. *Cereal Food World,* **44**, 590–596.

McCleary, B.V. & Blakeney, A.B. (1999) Measurement of inulin and oligofructan. *Cereal Food. World*, **44**, 398–406.

McCleary, B.V. & Codd, R. (1991) Measurement of (1-3)(1-4)-β-D-glucan in barley and oats: a streamlined enzymic procedure. *Journal of the Science of Food and Agriculture*, **55**, 303–312.

McCleary, B.V. & Sheehan, H. (1987) Measurement of cereal α-amylase: a new assay procedure. *Journal of Cereal Science*, **6**, 237–251.

McCleary, B.V. & Glennie-Holmes, M. (1985) Enzymic quantification of (1-3)(1-4)-β-D-glucan in barley and malt. *Journal of the Institute of Brewing*, **91**, 285–295.

McCleary, B.V., Bouhet, F. & Driguez,H. (1991) Measurement of amyloglucosidase using p-nitrophenyl β-maltoside as substrate. *Biotechnology Techniques*, **5**, 255–258.

McCleary, B.V., Gibson, T.S. & Mugford, D.C. (1997) Measurement of total starch in cereal products by amyloglucosidase – alpha-amylase method. Collaborative study. *Journal of the Association of Official Analytical Chemists*, **80**, 571–579.

McCleary, B.V., Murphy, A. & Mugford, D.C. (2000) Measurement of total fructan in food by enzymic/spectrophotometric method: collaborative study. *Journal of the Association of Official Analytical Chemists*, **83**, 356–364.

Megazyme (1999) Assay of *endo*-protease using Azo-Casein. Booklet ACS2/99. Megazyme International Ireland Ltd, Bray, Ireland.

Mongeau, R., Scott, F.W. & Brassard, R. (1999) Definition and analysis of dietary fibre. In: *Complex Carbohydrates in Food.* (eds S.S. Cho, L. Prosky & M. Dreher), pp. 305–316. Marcel Dekker Inc., New York.

Muir, J.G. & O'Dea, K. (1993) Validation of an *in vitro* assay for predicting the amount of starch that escapes digestion in the small intestine of humans. *American Journal of Clinical Nutrition,* **57**, 540–546.

Muir, J.G., Young, G.P., O'Dea, K., Cameron-Smith, D., Brown, I.L. & Collier, G.R., (1993) Resistant starch: the neglected 'dietary fibre'? Implications for health. *Dietary Fibre Bibliography and Reviews*, **1**, 33–47.

Pontis H.G. (1990) Fructans. In: *Methods in Plant Biochemistry.* (eds P.M. Dey & J.B. Harbourne), pp. 353–369. Academic Press, New York.

Prosky, L., Asp, N.G., Furda, I., DeVries, J.W., Schweizer, T.F. & Harland, B.F. (1985) Determination of total dietary fibre in foods and food products: collaborative study. *Journal of the Association of Official Analytical Chemists*, **68**, 677–679.

Prosky, L., Asp., N.-G., Schweizer, T.F., DeVries, J.W. & Furda,I. (1988) Determination of insoluble, soluble and total dietary fibre in foods and food products: interlaboratory study. *Journal of the Association of Official Analytical Chemists,* **71**, 1017–1023.

Quemener, B., Thibault, J.-F. & Coussement, P. (1993) Determination of inulin and oligofructose in food products, and integration in the AOAC method for measurement of total dietary fibre. *Lebensmittel Wissenschaft und Technologie*, **27**, 125–132.

Quemener, B., Thibault, J.-F. & Coussement,P. (1997) Integration of inulin determination in the AOAC method for measurement of total dietary fibre. *International Journal of Biological Macromolecules,* **21**, 175–178.

Quigley M.E. & Englyst, H.N. (1994) Determination of the uronic acid constituents of non-starch polysaccharides by high-performance liquid chromatography with pulsed amperometric detection. *Analyst*, **119**, 1511–1518.

Somogyi, M. (1952) Notes on sugar determination. *Journal of Biological Chemistry,* **195**, 19–23.

Theander, O., Aman, P., Westerlund, E., Andersson, R. & Pettersson, D. (1995). Total dietary fibre determined as neutral sugar residues, uronic acid residues and Klason lignin (The Uppsala method): a collaborative study. *Journal of the Association of Official Analytical Chemists*, **78**, 1030–1044.

Trowell, H.C. (1974) Definitions of fibre. *Lancet*, **i**, 503.