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RESEARCH ARTICLE

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# Measurement of available carbohydrates, digestible, and resistant starch in food ingredients and products

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## Abstract

**Background and objectives:** The importance of selectively measuring available and unavailable carbohydrates in the human diet has been recognized for over 100 years. The levels of available carbohydrates in diets can be directly linked to major diseases of the Western world, namely Type II diabetes and obesity. Methodology for measurement of total carbohydrates by difference was introduced in the 1880s, and this forms the basis of carbohydrate determination in the United States. In the United Kingdom, a method to directly measure available carbohydrates was introduced in the 1920s to assist diabetic patients with food selection. The aim of the current work was to develop simple, specific, and reliable methods for available carbohydrates and digestible starch (and resistant starch). The major component of available carbohydrates in most foods is digestible starch.

**Findings:** Simple methods for the measurement of rapidly digested starch, slowly digested starch, total digestible starch, resistant starch, and available carbohydrates have been developed, and the digestibility of phosphate cross-linked starch has been studied in detail. The resistant starch procedure developed is an update of current procedures and incorporates incubation conditions with pancreatic α-amylase (PAA) and amyloglucosidase (AMG) that parallel those used AOAC Method 2017.16 for total dietary fiber. Available carbohydrates are measured as glucose, fructose, and galactose, following complete and selective hydrolysis of digestible starch, maltodextrins, maltose, sucrose, and lactose to glucose, fructose, and galactose. Sucrose is hydrolyzed with a specific sucrase enzyme that has no action on fructo-oligosaccharides (FOS).

**Conclusions:** The currently described "available carbohydrates" method together with the total dietary fiber method (AOAC Method 2017.16) allows the measurement of all carbohydrates in food products, including digestible starch.

**Significance and novelty:** This paper describes a simple and specific method for measurement of available carbohydrates in cereal, food, and feed products. This is the first method that provides the correct measurement of digestible starch and sucrose in the presence of FOS. Such methodology is essential for accurate labeling of food products, allowing consumers to make informed decisions in food selection.

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**KEYWORDS**
available carbohydrates, dietary fiber, digestible starch, Fibersym®, resistant starch

## 1 | INTRODUCTION

Available carbohydrate has been defined as the sum of free sugars (glucose, fructose, galactose, sucrose, maltose, lactose, and oligosaccharides) and complex carbohydrates (dextrins, starch, and glycogen). These are carbohydrates that are digested and absorbed, and are glucogenic in humans (Anon, 2003). In the "FAO/WHO scientific update on carbohydrates in human nutrition" (Mann et al., 2007), it was proposed that the term "dietary fiber" should be limited to polysaccharides that are intrinsic to the plant cell wall, and the methods for measuring dietary fiber are those which can reliably quantify the component polysaccharides. Direct chemical measurement was recommended over empirical gravimetric methods for this purpose. Resistant starch (RS) was not considered to be dietary fiber. In the food composition tables amassed in McCance and Widdowson's "The Composition of Foods" (Anon, 2003), dietary fiber is determined as nonstarch polysaccharides (NSP), as defined by Englyst, Wiggins & Cummings (1982), Englyst and Hudson (1987) and Englyst and Cummings (1988). However, in the definition for dietary fiber adopted in June 2009 by the Codex Alimentarius Commission (CAC) (2010), the definition includes carbohydrate polymers that are not hydrolyzed by the endogenous enzymes in the small intestine of humans and thus includes RS.

In most plant-based foods, the major contributor to available carbohydrates is digestible starch. Digestible starch was subcategorized by Englyst, Kingman, and Cummings (1992) as rapidly digested starch (RDS; that starch hydrolyzed by saturating levels of pancreatic α-amylase [PAA] and amyloglucosidase [AMG] in 20 min) and slowly digested starch (SDS, starch digested by PAA and AMG between 20 and 120 min). The remainder, the nonhydrolyzed starch, was termed RS. However, several studies (Deiteren et al., 2010; Geboes, Luypaerts, Rutgeerts, & Verbeke, 2003; Geypens et al., 1999; Miller et al., 1997; Sadik, Abrahamsson, Bjornsson, Gunnarsdottir, & Stotzer, 2003; Stotzer & Abrahamsson, 2010; Zarate et al., 2010) indicate that the time of residence of food in the human small intestine is approximately 4 hr (not 2 hr). For this reason, we introduce the term "total digestible starch, TDS," being starch which is digested by saturating levels of PAA and AMG at 37°C and pH 6.0 within 4 hr. The starch not hydrolyzed in 4 hr is termed RS. Consistent with the Codex Alimentarius definition of dietary fiber, this RS is included as part of dietary fiber.

A method for measurement of dietary fiber, generally consistent with the Codex definition, was published in 2007 (McCleary, 2007), and this method, following extensive interlaboratory evaluation, was adopted as AOAC Methods 2009.01 and 2011.25, and AACCI Methods 32-45.01 and 32-50.01 (McCleary et al., 2010). Subsequently, various limitations of this method were identified, particularly the fact that an incubation time of 16 hr was employed, which was quite correctly considered not to be physiologically relevant. In the development of AOAC Methods 2009.01 and 2011.25, an incubation time of 16 hr was chosen to maintain consistency with the Official Method for measuring resistant starch (AOAC Method 2002.01; AOAC, 2012) and several other published methods (Akerberg, Liljeberg, Granfeldt, Drews, & Bjorck, 1998; Champ, 1992, Champ, Martin, Noah, & Gratas, 1999; Faisant et al., 1995; Goni, Garcia-Diz, Manas, & Saura-Calixto, 1996; Muir & O'Dea, 1992). In response to this limitation, the integrated TDF method was modified by reducing incubation time with PAA/AMG from 16 to 4 hr, and the enzyme concentrations appropriately increased to ensure that the resistant starch values obtained for a number of reference materials were in line with those obtained with AOAC Methods 2002.02 (McCleary, McNally, & Rossiter, 2002) and 2009.01 as well as ileostomy data (Champ et al., 1999). This update (McCleary, Sloane & Draga, 2015) was successfully subjected to interlaboratory evaluation under the auspices of AOAC International and ICC to become AOAC Method 2017.16 and ICC Method 185 (McCleary 2018; McCleary, Cox, Ivory, & Delaney, 2018).

In the current paper, the methodology employed in AOAC Method 2017.16 has been adapted to allow the specific measurement of digestible and resistant starch and available carbohydrates. Special reference has been directed to the measurement of phosphate cross-linked starch ($RS_4$). In this methodology, available carbohydrates are defined as glucose, fructose, galactose, maltose, lactose, sucrose, maltodextrins, and total digestible starch. Note that starch is measured as digestible starch rather than total starch, and sucrose is measured specifically in the presence of fructo-oligosaccharides (FOS) by hydrolysis with a sucrase enzyme that has no action on FOS.

## 2 | MATERIALS AND METHODS

### 2.1 | Materials

#### 2.1.1 | Chemicals and reagents

D/L-Maleic acid (M-0375), bovine serum albumin (A-2153), dimethyl sulphoxide (D-8779), and sodium azide (S-8032) were from Sigma-Aldrich Ireland Ltd. Acetic acid (glacial)

GR, sodium hydroxide, and calcium chloride ($CaCl_2.2H_2O$) were from Merck. Amyloglucosidase (AMG: E-AMGFR and E-AMGDF), pancreatic α-amylase (PAA: E-PANAA), PAA/AMG mixture (PAA 40 KU/g plus AMG 17 KU/g; E-PAAMG), heat-stable α-amylase (E-BLAAM), thermostable α-amylase (E-BSTAA), protease (E-BSPRT), Total Starch assay kit (K-TSTA), Resistant Starch assay kit (K-RSTAR), Resistant Starch (Rapid) assay kit (K-RAPRS), Digestible Starch/Resistant Starch assay kit (K-DSTRS), Available Carbohydrates assay kit (K-AVCHO), α-Amylase assay kit (Ceralpha®; K-CERA), Rapid Integrated TDF assay kit (K-RINTDF), Amberlite FPA53 ($OH^-$; G-AMBOH), and Amberlite 200C ($H^+$; G-AMBH) were obtained from Megazyme.

### 2.1.2 | Pure starch samples

Regular maize starch (RMS Lot 60401) was from Penford Australasia. Hylon VII® (Ref. 98GH8401), Novelose 330® (Ref. AH17529), and Novelose 240® (Ref. 96LF10063) were from National Starch and Chemical Company. These companies are now part of Ingredion. Native potato starch was from Avebe (Foxhol, The Netherlands). ActiStar® (enzyme-modified tapioca/cassava starch; US Patent 6,043,229) was from Cerestar (now Cargill Belgium). Potato amylose (A-9262), wheat starch (S-5127), and ACS Soluble starch (S-9765) were from Sigma Chemical Company.

### 2.1.3 | Processed food and breakfast cereals

Uncle Ben's Ready Rice, white, extra was obtained from Professor William Park, Texas A & M University, College Station, Texas, USA. Brennans wholemeal bread, Heinz® baked beans, Kellogg's® corn flakes, Kellogg's® All Bran®, Weetabix®, Kellogg's® Special K®, Kellogg's® Sugar Frosties®, tinned butter beans, tinned chickpeas, tinned garden peas, tinned kidney beans, semigreen banana, Ryvita® crackers, and Roma® macaroni pasta were obtained from a local supermarket.

### 2.1.4 | Beans and fresh vegetables

Sweet corn, potatoes, garden peas, red kidney beans, chickpeas, fresh cabbage, broccoli, cauliflower, swede, red pepper, mushroom, ripe banana, uncooked red kidney beans and soybeans, red onion, celery, sweet potato, semiripe banana, carrots, and potato were obtained from a local supermarket. Potato was cooked in boiling water for 30 min, mashed, and freeze-dried. All fresh vegetables were sliced into thin sections, freeze-dried, milled to pass a 0.5-mm screen, and stored in Duran® airtight bottles at room temperature. Canned beans and vegetables were poured onto a strainer and washed with demineralized water, freeze-dried, and milled to pass a 0.5-mm screen. Dry breakfast cereals were milled to pass a 0.5-mm screen and stored in airtight Duran® bottles.

## 2.2 | Analytical methods

### 2.2.1 | Preparation of test samples

High-moisture containing samples (>25%) were freeze-dried. Samples ca. 50 g were ground in a grinding mill, to pass a 0.5-mm sieve. All materials were transferred to a wide-mouthed plastic jar, sealed, and mixed well by shaking and inverting and then stored in the presence of a desiccant. Food samples were collected and prepared as "intended to be eaten"; that is, pasta and potatoes were cooked. High-fat-containing samples such as chocolate peanuts, chocolate cookies, and jam tarts were homogenized using a Nutri-Bullet homogenizer. A sample of the homogenized material (approximately 2 g, weighed accurately) was transferred to an ANKOM® filter bag, and the bags were sealed. The filter bags were dried in an oven at 105°C before being placed into a desiccator to cool. The weight of the bag plus sample was measured and recorded. The samples were then defatted using the ANKOM defatting apparatus at 60°C over 90 min with petroleum ether. The recovered bags were air-dried for 15 min in a fume cupboard and then dried in an oven at 105°C for 30 min.

### 2.2.2 | Measurement of enzyme activities

α-Amylase activity in PAA was measured using the Ceralpha® assay procedure employing benzylidene blocked *p*-nitrophenyl maltoheptaoside in the presence of excess levels of thermostable α-glucosidase. Incubations were performed in sodium maleate buffer at pH 6.9 and 40°C as described in the Ceralpha® kit booklet (Megazyme K-CERA; AOAC Official Method 2002.01). One unit (U) of enzyme activity is defined as the amount of enzyme that releases one μmole of *p*-nitrophenol per minute under the defined assay procedure. The α-amylase activity reported is that measured at the optimal pH of 6.9. However, incubations for the measurement of digestible starch, resistant starch, and available carbohydrates were performed at pH 6.0. α-Amylase activity at pH 6.0 is ~77% of that at pH 6.9 (McCleary & Monaghan, 2002). AMG was assayed by incubating 0.2 ml of suitably diluted enzyme in 100 mM sodium acetate buffer (pH 4.5) with 0.5 ml of soluble starch (10 mg/ml) in 100 mM sodium acetate buffer (pH 4.5) at 40°C. At various time intervals, reaction tubes were heated to ~100°C in a boiling water bath to terminate the reaction and released glucose was measured using GOPOD reagent (Glucose assay kit; Megazyme K-GLUC). One unit of AMG is defined as the amount of enzyme required to release one μmole of glucose per minute at pH 4.5 and 40°C. When in admixture with PAA, AMG was

assayed using AMG Assay Reagent (Megazyme R-AMGR3) and units of activity on starch were calculated using a conversion factor. The AMG activity reported is that measured at the optimal pH of 4.5. However, incubations for the measurement of digestible starch, resistant starch, and available carbohydrates were performed at pH 6.0. AMG activity at pH 6.0 is ~20% of that at pH 4.5 (McCleary & Monaghan, 2002).

## 2.3 | Measurement of RS and DS using the rapid RS method

Resistant starch was measured according to AOAC Method 2002.02/AACC Method 32-40.01 and also using the rapid RS method described here. In this latter procedure, samples of finely milled (0.5 mm) cereal or food samples (~100 mg weighed accurately) were weighed into 16.5 × 101 mm, 13-ml polypropylene tubes, and the tubes were tapped gently to ensure that all samples fell to the bottom of the tube. For wet samples such as minced canned beans or food product, a sample size of approximately 0.5 g (weighed accurately) was analyzed. An aliquot (3.5 ml) of sodium maleate buffer (pH 6.0) containing 2 mM calcium chloride was added, and the contents mixed thoroughly on a vortex mixer for 5 s and the tube placed in a water bath at 37°C for 5 min to allow the contents to equilibrate to temperature. An aliquot (0.5 ml) of PAA/AMG solution (0.4 KU PAA plus 0.17 KU AMG) was added to each tube, and the tubes were capped tightly and attached horizontally, aligned in the direction of motion (Figure 1) in a shaking water bath set at 37°C (Note: If an $(NH_4)_2SO_4$ suspension of this enzyme preparation [PAA, 2 KU/ml; AMG, 0.83 KU/ml in 50% *w*/*v* ammonium sulfate] was used, the sample was suspended in 3.8 ml of sodium maleate buffer and 0.2 ml of enzyme suspension was added.). Tubes were incubated at 37°C with continuous shaking (200 strokes/min) for exactly 4 hr. The tubes were removed from the water bath one at a time, ethanol or IMS (industrial methylated spirits; 4.0 ml, 95% *v*/*v*) added, the tubes were capped, and the contents were stirred vigorously on a vortex mixer. After removal of caps, the tubes were centrifuged at 3,250 *g* (approx. 3,250 relative centrifugal force; rcf) for 10 min. Immediately after the centrifuge stopped, the supernatant solution was carefully decanted (ensuring that the pellets were not disturbed) and stored for the determination of DS. The pellets were re-suspended in 2 ml of 50% *v*/*v* aqueous ethanol or IMS and mixed vigorously on a vortex mixer. A further 6 ml of 50% *v*/*v* aqueous ethanol or IMS was then added to the tube, the tube was capped, and the contents were mixed thoroughly by inversion. Tubes were tapped so that all liquid was removed from the caps. Caps were then removed, and the tubes were centrifuged at 3,250 *g* for 10 min. The supernatant solutions were then carefully decanted and added to the original supernatant. The residue was re-suspended in 8 ml of 50% *v*/*v* aqueous ethanol or IMS and centrifuged, and the supernatant decanted and added to the first two supernatant solutions. The tubes containing the residue were inverted on absorbent paper to remove excess liquid while ensuring that the pellets were not dislodged.


**FIGURE 1** Attachment of 13-ml polypropylene tubes to a polypropylene tube holder in a Grant OLS 200 water bath [Color figure can be viewed at wileyonlinelibrary.com]

### 2.3.1 | Determination of RS

A magnetic stirrer bar (6 × 12 mm) and 2 ml of ice-cold 1.7 M NaOH were added to each tube, and the pellets were re-suspended (and RS dissolved) by stirring for approx. 20 min in an ice/water bath over a magnetic stirrer (Figure 2). Sodium acetate buffer (8 ml, 1.0 M, pH 3.8) containing calcium chloride (5 mM) was added to each tube while stirring. AMG (0.1 ml, 3,300 U/ml) was immediately added; the tubes were mixed well and placed in a water bath at 50°C and incubated for 30 min with intermittent mixing on a vortex mixer. For samples containing <10% RS content, centrifuge aliquots of the undiluted solutions at 8,000 *g* for 5 min in a microfuge. The final volume in the tube (before removal of an aliquot for centrifugation) was approximately 10.3 ml. However, this volume varied, particularly when wet samples were analyzed, and appropriate allowances for the final volumes were made in the calculations. For samples containing >10% RS content, the contents of the tubes were quantitatively transferred to 100-ml volumetric flask using a water wash bottle. The volume was adjusted to 100 ml with distilled water and mixed

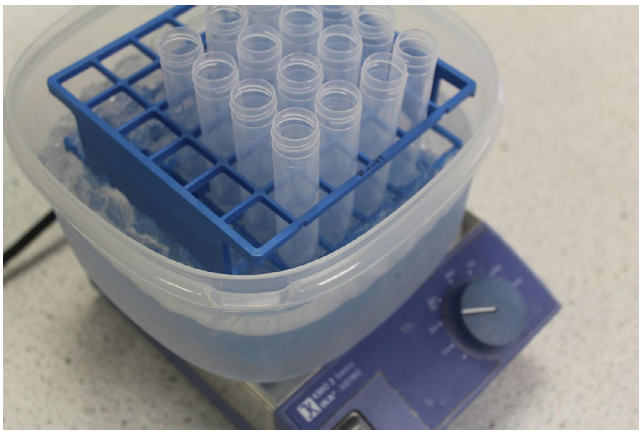

**FIGURE 2** Arrangement of tubes in an ice-water bath over a magnetic stirrer for dissolution of resistant starch with 1.7 M NaOH [Color figure can be viewed at wileyonlinelibrary.com]

well. Aliquots of all solutions were centrifuged at 8,000 *g* for 5 min. Duplicate aliquots (0.1 ml) of all solutions were transferred to the bottoms of glass test tubes (16 × 100 mm), and 3.0 ml of GOPOD reagent was added with mixing, and the tubes were incubated at 50°C for 20 min. A reagent blank solution was prepared by mixing 0.1 ml of 100 mM acetic acid (pH 4.5) with 3.0 ml of GOPOD reagent. Glucose standards (in quadruplicate) were prepared by mixing 0.1 ml of glucose solution (1 mg/ml) with 3.0 ml of GOPOD reagent and incubating at 50°C for 20 min. Absorbance of each solution was measured at 510 nm against the reagent blank.

Content of RS was calculated as follows:

$$\begin{aligned}&\text{RS (g/100g)}\\&=\Delta A\times F\times \text{EV}/0.1\times 1/1000\times 100/W\times 162/180\\&=\Delta A\times F\times \text{EV}/W\times 0.9\end{aligned}$$

where $\Delta A$ = absorbance of sample solution read against reagent blank, $F$ = factor to convert absorbance values to μg glucose (100 μg glucose divided by the absorbance value obtained for 100 μg of glucose), EV = sample extraction volume (10.3 or 100 ml), 0.1 = volume of sample analyzed, 1/1,000 = conversion from μg to mg, 100/W = conversion to 100 mg sample, W = sample weight in mg, 162/180 = factor to convert from free glucose, as determined, to anhydro-glucose, as occurs in starch.

Calculations are simplified using a Megazyme MegaCalc™ Excel® based calculator (RAPRS) in Supporting Information.

### 2.3.2 | Determination of DS

The combined supernatants were adjusted to 100 ml with 100 mM sodium acetate buffer (pH 4.5), and an aliquot (2.0 ml) was centrifuged at 8,000 *g* for 5 min. To determine digestible starch, duplicate aliquots (0.1 ml) were transferred to the bottoms of two 16 × 100 mm tubes, 0.1 ml of AMG (100 U/ml) in 100 mM sodium acetate buffer (pH 4.5) was added, and the tubes were incubated at 50°C for 30 min. GOPOD reagent (3.0 ml) was added, and the tubes were incubated at 50°C for 20 min. A reagent blank solution was prepared by mixing 0.2 ml of 100 mM acetic acid (pH 4.5) with 3.0 ml of GOPOD reagent. Glucose standards (in quadruplicate) were prepared by mixing 0.1 ml of glucose solution (1 mg/ml) with 0.1 ml of 100 mM sodium acetate buffer (pH 4.5) and 3.0 ml of GOPOD reagent and incubating at 50°C for 20 min. Absorbance of each solution was measured at 510 nm against the reagent blank. The total carbohydrate content of the solutions was determined on 50 μl aliquots using the phenol-sulfuric acid procedure of DuBois, Gilles, Hamilton, Rebers, and Smith (1956).

Calculate DS (% *w*/*w*, "as is" basis) in test samples as follows:

$$\begin{aligned}&\text{DS (g/100g sample)}\\&=\Delta A\times F\times \text{EV}/0.1\times 1/1000\times 100/W\times 162/180\\&=\Delta A\times F/W\times 0.9\end{aligned}$$

where $\Delta A$ = absorbance (reaction) read against the reagent blank, $F$ = conversion from absorbance to μg (the absorbance obtained for 100 μg of glucose in the GOPOD reaction is determined), ($F$ = 100 [μg of glucose] divided by the GOPOD absorbance for this 100 μg of glucose), EV = sample extraction volume (ml) = 100. 0.1 = volume of sample analyzed, 1/1,000 = conversion from μg to mg, 100/$W$ = conversion to 100 mg of sample, $W$ = sample weight in mg; 162/180 = factor to convert from free glucose, as determined, to anhydro-glucose as occurs in starch.

### 2.4 | Measurement of phosphate cross-linked starch ($RS_4$)

Phosphate cross-linked starch was measured by several methods, the method of Shukri, Zhu, Seib, Maningat, and Shi (2015) and Shi, Sun, and Shi (2019) and other methods as described here. In the modified Shukri et al. (2015) and Shi et al. (2019) methods as described here, the initial incubation with PAA and AMG was performed under the conditions of AOAC Method 2017.16 (PAA 100 U/ml; AMG 42 U/ml; Table 1) for 4 hr, instead of that reported by Shukri et al. (2015) and Shi et al. (2019) (PAA 50 U/ml; AMG 3 U/ml) for 2 hr. The RS fraction was then recovered and digested according to the particular procedures described by Shukri et al. (2015) or Shi et al. (2019). In the Shukri et al. (2015) method, the RS fraction (e.g., from Fibersym® [$RS_4$]) was recovered by precipitation with ethanol, centrifugation, washing the residue with ethanol, and incubated with protease followed by thermostable α-amylase (*Bacillus* sp.; 200 U) at 100°C for 30 min in 8 ml of 100 mM sodium acetate buffer, pH 5 containing 5 mM $CaCl_2$. A second amount of α-amylase

**TABLE 1** A comparison of incubation conditions used in the resistant starch, digestible starch, available carbohydrates, and dietary fiber assay procedures[a]

| Component measured | Method, incubation time, and catalogue number | Sample weight | Buffer volume | Pancreatic α-amylase | | Amyloglucosidase | |
|---|---|---|---|---|---|---|---|
| | | mg | ml | U/ml of assay solution | K Units per assay | Units/ml of assay solution | K Units per assay |
| Resistant starch[b] | AOAC Method 2002.02<br>16 hr (K-RSTAR) | ~100 | 4 | 50 | 0.2 | 3 | 0.014 |
| Total dietary fiber[b] | AOAC Method 2009.01/AACC Method 32-45.01<br>16 hr (K-INTDF)[c] | ~1,000 | 40 | 50 | 2.0 | 3 | 0.14 |
| Total dietary fiber[b] | AOAC Method 2011.25<br>AACC Method 32-50.01<br>16 hr (K-INTDF) | ~1,000 | 40 | 50 | 2.0 | 3 | 0.14 |
| Resistant starch | Rapid resistant starch<br>4 hr (K-RAPRS) | ~100 | 4 | 100 | 0.4 | 42 | 0.17 |
| Digestible/resistant starch | Digestible/resistant starch method<br>4 hr (K-DSTRS) | ~500 | 20 | 100 | 2.0 | 42 | 0.85 |
| Available carbohydrates | Available carbohydrates<br>4 hr (K-AVCHO) | ~500 | 20 | 100 | 2.0 | 42 | 0.85 |
| Total dietary fiber | AOAC Method 2017.16<br>4 hr (K-RINTDF) | ~1,000 | 40 | 100 | 4.0 | 42 | 1.7 |

[a]In all cases, incubations were performed in 50 mM sodium maleate buffer (pH 6.0) containing 2 mM calcium chloride.
[b]Sodium azide (0.02% *w*/*v*) was included in the buffer in these procedures.
[c]Megazyme catalogue numbers.

(200 U) was added, and the sample was incubated for a further 30 min. The solution was cooled to 50°C, 132 U of AMG added, and the solutions were incubated for 1 hr. Released glucose was analyzed with GOPOD reagent and RS calculated according to Shukri et al. (2015). In the "improved" in vitro assay of Shi et al. (2019), the RS residue was suspended in 2 M KOH and stirred at room temperature for 4 hr. The solution was neutralized with HCl, ethanol was added, and the residue was recovered by centrifugation (the supernatant was recovered for determination of DS.). The residue was suspended in 8 ml of 100 mM sodium acetate buffer (pH 5.0), thermostable α-amylase (200 U) was added, and the solution was incubated at 100°C for 30 min with intermittent vigorous stirring. This α-amylase treatment was then repeated a second time. The solution was cooled to 50°C, 132 U of AMG added, and the solution incubated at 50°C for 1 hr. Released glucose was analyzed with GOPOD reagent and RS calculated according to Shi et al. (2019).

In the current procedure, samples (~100 mg weighed accurately) were weighed into 16.5 × 101 mm, 13-ml polypropylene tubes, and an aliquot (3.5 ml) of sodium maleate buffer (pH 6.0) plus 2 mM $CaCl_2$ was added, and the contents mixed thoroughly on a vortex mixer for 5 s and the tube placed in a water bath at 37°C for 5 min to allow the contents to equilibrate to temperature. An aliquot (0.5 ml) of PAA/AMG solution (0.4 KU PAA plus 0.17 KU AMG) was added to each tube, and the tubes were capped tightly and attached horizontally, aligned in the direction of motion (Figure 1) in a shaking water bath set at 37°C. Tubes were incubated at 37°C with continuous shaking (200 strokes/min) for exactly 4 hr. Ethanol or IMS (4.0 ml, 95% *v*/*v*) was added to each tube, and the contents were stirred vigorously on a vortex mixer. Tubes were centrifuged, and the initial supernatant was stored in a sealed tube for determination of digestible starch. The residue containing the RS was recovered and washed as in the standard rapid RS method, and the supernatants from the two washings with aqueous ethanol were added to the initial supernatant and used for the measurement of digestible starch. Several procedures were evaluated for the dissolution and hydrolysis of the starch in the residue fraction, namely.

a. the residue was suspended in 8 ml of 100 mM sodium acetate buffer (pH 5.0), 0.1 ml of thermostable α-amylase (200 U, E-BSTAA) was added, and the tubes were incubated at 100°C for 30 min. The tubes were then cooled to 50°C, 0.1 ml of AMG (330 U) was added, and the tubes were incubated at 50°C for 30 min. Volumes were adjusted to 100 ml with distilled water and the contents thoroughly

mixed. Aliquots (2.0 ml) were centrifuged at 8,000 g for 5 min in a microfuge, and 0.1 ml aliquots removed for the determination of glucose using GOPOD reagent and determination of RS according to the procedure used in the RS (rapid) method. Digestible starch was determined using the same procedure for the determination of digestible starch in the RS (rapid) method.

b. The residue was suspended in 2 ml of sodium hydroxide (1.7 M) and vigorously mixed on a vortex mixer, and the tubes were incubated at 50°C for 15 min (incubation times of 30, 60, and 120 min were also evaluated). The solutions were neutralized by addition of 8 ml of sodium acetate buffer (600 mM, pH 3.8), 0.1 ml of thermostable α-amylase (200 U, E-BSTAA) was added, and the tubes were incubated at 100°C for 30 min. The tubes were then cooled to 50°C and 0.1 ml of AMG (330 U) was added, and the solutions incubated at 50°C for 30 min. Volumes were adjusted to 100 ml with distilled water and the contents thoroughly mixed. Aliquots were removed for the measurement of glucose and determination of RS according to example "a." Digestible starch was determined using the same procedure for the determination of digestible starch in the RS (rapid) method.
c. The residue was suspended in 2 ml of ice-cold sodium hydroxide (1.7 M) and vigorously mixed on a vortex mixer, and the tubes were incubated at ~4°C with stirring for 30 min. The solutions were neutralized by addition of 8 ml of sodium acetate buffer (600 mM, pH 3.8), and all other steps were as described in example "b."
d. The residue was suspended in 2 ml of sodium hydroxide (1.7 M) and vigorously mixed on a vortex mixer, and the tubes were incubated at 4°C for 20 min. The solutions were neutralized by addition of 8 ml of sodium acetate buffer (1.2 M, pH 3.8), 0.1 ml of AMG (330 U) was added, and the tubes were incubated at 50°C for 30 min. Volumes were adjusted to 100 ml with distilled water and the contents thoroughly mixed. Aliquots were removed for the measurement of glucose and determination of RS according to example "a." Digestible starch was determined using the same procedure for the determination of digestible starch in the RS (rapid) method. This is the procedure employed to dissolve RS in the rapid RS assay procedure.

In other experiments, the total starch content of phosphate cross-linked and other starches was determined by directly using the dissolution and hydrolysis conditions for the RS residue described in examples "a"–"d" above.

The total carbohydrate content of the digestible starch and hydrolyzed resistant starch fractions was determined by analyzing an aliquot (50 µl) with the phenol-sulfuric acid procedure of DuBois et al. (1956).

## 2.5 | Chromatographic separation of the carbohydrates present in the hydrolysate of Fibersym®

Twenty separate samples of Fibersym® (1.00 g each) were incubated with PAA/AMG according to AOAC Method 2017.16. After 4 hr, the pH was adjusted to ~8.2 according to the procedure, and the solutions heated to ~95°C to inactivate PAA and AMG. Four volumes of ethanol (160 ml) was added to each of the twenty containers and mixed thoroughly. After standing at room temperature overnight, the contents of all containers were pooled and centrifuged at 24,000 *g* for 20 min. The supernatants (original supernatants) were carefully decanted, combined, and concentrated by rotary evaporation. The residues were pooled into two 400-ml centrifuge containers and suspended in ~200 ml of 80% *v*/*v* ethanol in water (in each container) and recovered by centrifugation. This procedure was repeated once more. Each of the supernatants was pooled with the original supernatant and concentrated. Carbohydrate in the supernatant fraction (DS) was concentrated to ~30 mg/ml, and aliquots (16 ml) fractionated on a column (5 × 95 cm) of Bio-Gel P-2, Extra Fine (Bio-Rad Laboratories) in distilled water at 60°C. Fractions of 20 ml were collected, and aliquots analyzed for total carbohydrate using the phenol-sulfuric acid procedure (DuBois et al., 1956). The fractions shown in Figure 5 were collected and concentrated (where necessary) by rotary evaporation to a carbohydrate concentration of ~0.5 mg/ml. These solutions were analyzed for free glucose by incubating aliquots (0.1 ml) plus 0.1 ml of sodium acetate buffer with 3.0 ml of GOPOD reagent at 50°C for 20 min. The absorbance was measured against a blank solution containing 0.2 ml of 100 mM sodium acetate buffer (4.5) plus 3.0 ml of GOPOD reagent. Glucose standard solutions were prepared by incubating glucose (0.1 ml, 1.0 mg/ml) plus 0.1 ml of 100 mM sodium acetate buffer (pH 4.5) with 3.0 ml of GOPOD reagent at 50°C for 20 min concurrently with the sample solutions. Hydrolysis by AMG was determined by incubating sample aliquots (0.1 ml, ~0.5 mg/ml) in 100 mM sodium acetate buffer (pH 4.5) with 0.1 ml of AMG (10 U) for 30 min at 50°C with 3.0 ml of GOPOD reagent. Total carbohydrate concentration of the samples was determined on 0.1 ml aliquots using the phenol-sulfuric acid procedure (DuBois et al., 1956).

The combined residues from alcohol precipitation of the PAA/AMG incubation mixtures (the RS fractions) were suspended in 40 ml of 1.7 M NaOH, heated at 50°C for 30 min, and then neutralized by addition of 160 ml of 600 mM sodium acetate buffer (pH 3.8) plus 5 mM $CaCl_2$; the pH was ~5.3. Thermostable α-amylase (2 ml, 2,000 U/ml) was added, and the solution incubated at ~100°C for 30 min. The temperature was reduced to 50°C and 2 ml of

AMG (3,300 U/ml) added, and the solutions incubated for 30 min at 50°C and then at ~100°C for 10 min to inactivate the AMG. The solution was concentrated to ~20 mg carbohydrate/ml, centrifuged at 29,000 *g* for 15 min to remove a very light precipitate (which on recovery represented 0.16% of total carbohydrate in the Fibersym® sample), and aliquots (16 ml) fractionated on a column (5 × 95 cm) of Bio-Gel P-2, Extra Fine (Bio-Rad Laboratories) in distilled water at 60°C. Fractions of 20 ml were collected, and aliquots analyzed for total carbohydrate using the phenol-sulfuric acid procedure (DuBois et al., 1956). Individual fractions were concentrated (where necessary) to ~0.5 mg/ml, and duplicate aliquots (0.1 ml) were analyzed for total carbohydrate by the phenol-sulfuric acid method. Separate aliquots (0.1 ml) were analyzed for free glucose by incubation with GOPOD reagent (3.0 ml) at 50°C for 20 min or were incubated with AMG (0.1 ml, 10 U) in 100 mM sodium acetate buffer (pH 4.5) for 30 min at 50°C and then with GOPOD reagent (3.0 ml) at 50°C for 20 min, and the absorbance at 510 nm measured against a reagent blank. Glucose standard solutions and blank solutions were run concurrently.

## 2.6 | Measurement of digestible (RDS, SDS, TDS) and resistant starch

Samples of finely milled (<0.5 mm) cereal or food samples (~0.5 g weighed accurately) were weighed into 30 × 84 mm (40 ml) polypropylene tubes, and the weight recorded. A 20 × 6 mm stirrer bar was added to each tube, the sample was wet with 0.5 ml of ethanol (95% *v*/*v*), and 17.5 ml of maleate buffer was added to each tube. The tubes were capped and placed in a special polypropylene holder (Figure 3) on a 2mag Mixdrive 15® submersible magnetic stirrer in a water bath and allowed to equilibrate to 37°C over 5 min with stirring at 170 rpm. An aliquot (2.5 ml) of PAA/AMG solution (PAA, 2 KU; AMG, 0.85 KU) was added, and the tubes were capped and incubated at 37°C with stirring at 170 rpm on the 2mag Mixdrive 15® submersible magnetic stirrer. If an $(NH_4)_2SO_4$ suspension of this enzyme preparation (PAA, 2 KU/ml; AMG, 0.83 KU/ml) was used, the sample was suspended in 19 ml of sodium maleate buffer and 1.0 ml of enzyme suspension was added.

### 2.6.1 | Determination of DS

Aliquots (1.0 ml) of the stirred reaction solution were removed using a positive displacement dispenser at 20 min (for determination of RDS), at 120 min (for determination of SDS; SDS = DS at 120 min − DS at 20 min), and at 240 min (for determination of TDS). These aliquots were immediately added to 20 ml of 50 mM acetic acid solution, and the tubes were capped and mixed thoroughly. These were stored at 4°C awaiting analysis. Aliquots (2 ml) of each solution were transferred to 2.0-ml polypropylene microfuge tubes and centrifuged at 8,000 *g* for 5 min. Duplicate aliquots (0.1 ml) were then transferred to the bottoms of 16 × 100 mm glass test tubes, 0.1 ml of AMG (10 U) in 200 mM sodium acetate buffer (pH 4.5) was added, and the tubes were incubated at 50°C for 30 min. GOPOD reagent (3.0 ml) was added, and the tubes were incubated at 50°C for 20 min. A reagent blank solution was prepared by mixing 0.2 ml of 200 mM acetic acid (pH 4.5) with 3.0 ml of GOPOD reagent and incubating at 50°C for 20 min. Glucose standards (in quadruplicate) were prepared by mixing 0.1 ml of glucose solution (1 mg/ml) with 0.1 ml of 200 mM sodium acetate buffer (pH 4.5) and 3.0 ml of GOPOD reagent and incubating at 50°C for 20 min. The absorbance of each solution was measured at 510 nm against the reagent blank.

Calculate DS (RDS, SDS & TDS; % *w*/*w*, "as is" basis) in test samples as follows:

$$\begin{aligned}&\text{DS (RDS, SDS or TDS) (g/100g sample)} = \Delta A \times F \times \text{EV}/W\\&\times D/0.1\times 100\times 1/1{,}000{,}000\times 100/W\times 162/180\\&= \Delta A\times F/W\times 0.38745.\end{aligned}$$

where $\Delta A$ = absorbance (reaction) read against the reagent blank after 20 min (RDS); after 120 min − 20 min (SDS); after 240 min (TDS), $F$ = conversion from absorbance to μg (the absorbance obtained for 100 μg of glucose in the GOPOD reaction is determined; $F$ = 100 [μg of glucose] divided by the GOPOD absorbance for this 100 μg of glucose), and EV = extraction volume (ml) = 20.5.

$W$ = "as is" weight of sample analyzed in g, that is, ~0.50 g (weighed accurately).

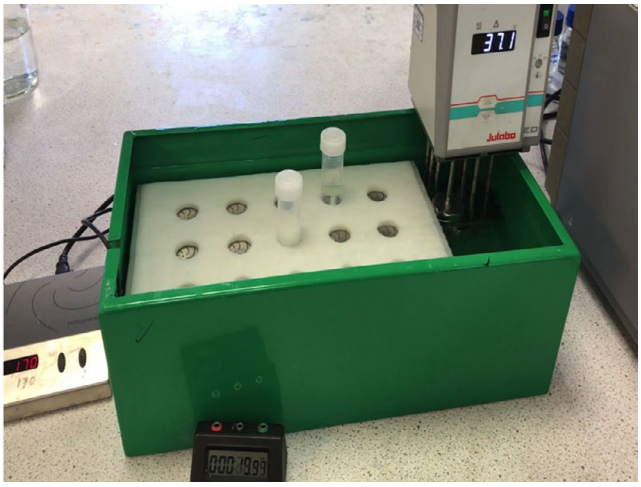


**FIGURE 3** Samples (~0.5 g) in 40 ml, 30 × 84 mm polypropylene tubes in a designed polypropylene tube holder (Megazyme cat. no. D-PPTH) [C (w)] on a 2mag Mixdrive 15® submersible magnetic stirrer in a custom-made water bath (Megazyme cat. no. D-TDFBTH). This arrangement allows stirring of 15 samples at controlled speed (170 rpm) and 37°C [Color figure can be viewed at wileyonlinelibrary.com]

$D$ = dilution of sample (21; 1.0 ml of sample added to 20 ml of dilute acetic acid).

0.1 = volume of sample analyzed. 100 = conversion to g/100 g. 1/1,000,000 = conversion from μg to g. 162/180 = factor to convert from free glucose, as determined, to anhydro-glucose as occurs in starch.

Calculations are simplified using a Megazyme MegaCalc™ Excel®-based calculator (DSTRS-DS) in Supporting Information.

### 2.6.2 | Determination of RS

Aliquots (4 ml) were removed from the stirring reaction solutions using a positive displacement dispenser after 240 min (4 hr) of incubation, and transferred to a 16.5 × 101 mm, 13-ml polypropylene tubes containing 4.0 ml of ethanol (95% *v*/*v*) or IMS. The tubes were capped, and the contents thoroughly mixed by repeated inversion. Tubes were centrifuged at 3,250 *g* for 10 min in a bench centrifuge, and the supernatant carefully decanted immediately after the centrifuge had stopped. Each pellet was re-suspended in 2 ml of 50% *v*/*v* aqueous IMS in water by stirring on a vortex mixer. Another 6 ml of 50% *v*/*v* aqueous IMS was then added to the tube, which was then capped, and the contents mixed on a vortex mixer. The tubes were centrifuged, and the pellets recovered by centrifugation. This process of suspension and centrifugation was repeated, and the supernatant again carefully decanted. Free liquid in the tube was removed by inverting the tubes on absorbent paper while ensuring that the pellet was not dislodged. A magnetic stirrer bar (6 × 12 mm) and 2 ml of cold 1.7 M NaOH were added to each tube, and the pellets were re-suspended (and the RS dissolved) by stirring the tube contents for approx. 20 min in an ice/water bath over a magnetic stirrer (Figure 2). The solutions were neutralized with 8 ml of 1.0 M sodium acetate buffer (pH 3.8) containing 5 mM $CaCl_2$, starch hydrolyzed, and glucose measured as described for rapid RS method.

Calculate RS (% *w*/*w*, on an "as is" basis) in test samples as follows:

$$\begin{aligned}&\text{RS (g/100g sample)}\\&= \Delta A \times F \times \text{EV}/4 \times \text{FV}/0.1 \times 1/1{,}000{,}000 \times 100/W \times 162/180\\&= \Delta A \times F/W \times \text{FV} \times 0.004613\end{aligned}$$

where $\Delta A$ = absorbance (reaction) read against the reagent blank, $F$ = conversion from absorbance to μg (the absorbance obtained for 100 μg of glucose in the GOPOD reaction is determined; $F$ = 100 [μg of glucose] divided by the GOPOD absorbance for this 100 μg of glucose), EV = extraction volume (ml) = 20.5, 4 = volume of solution taken from the reaction mixture for RS analysis, FV/0.1 = 0.1 ml aliquots taken from final volume (FV, either 100 or 10.3 ml) for the determination of glucose using GOPOD reagent, 1/1,000,000 = conversion from μg to g, 100/$W$ = conversion to g/100 g, $W$ = "as is" weight of sample analyzed in g (~0.50 g weighed accurately), and 162/180 = factor to convert from free glucose, as determined, to anhydro-glucose as occurs in starch.

Calculations are simplified using a Megazyme MegaCalc™ Excel®-based calculator (RAPRS-RS) in Supporting Information.

## 2.7 | Measurement of available carbohydrates

### 2.7.1 | Method

Samples of finely milled cereal or food samples (~0.5 g weighed accurately) were weighed into 30 × 84 mm (40 ml) polypropylene tubes and the weight recorded. Incubation with PAA/AMG for 4 hr was then performed exactly as described for "digestible and resistant starch" above. Aliquots (1.0 ml) were removed and added to 25 ml of 50 mM acetic acid as described and mixed thoroughly, and samples (2 ml) were centrifuged at 8,000 *g* for 5 min. Aliquots (0.1 ml) of this solution were analyzed for available carbohydrates (total digestible starch, maltodextrins, sucrose, lactose, glucose, and fructose). All incubations were performed as described in Figure 4. Reagents required for this determination are available in the Available Carbohydrates assay kit from Megazyme (cat. no. K-AVCHO). Aliquots (0.1 ml) of centrifuged solution in a spectrophotometer cuvette were incubated with 0.1 ml of a solution containing β-galactosidase (800 U/ml), sucrase (20 U/ml), and maltase (100 U/ml) in 50 mM sodium maleate buffer (pH 6.5) containing BSA (0.5 mg/ml) at 25°C for 20 min to hydrolyze lactose, sucrose, and maltose to monosaccharides. Distilled water (2.0 ml), imidazole buffer (0.1 ml, 2 M, pH 7.6) containing $MgCl_2$ (100 mM), and a solution (0.1 ml) of $NADP^+$ (20 mg/ml) plus ATP (40 mg/ml) were added and mixed, and the absorbance ($A_1$) measured at 340 nm after 3 min. An aliquot (20 μl) of hexokinase (420 U/ml) and glucose 6-phosphate dehydrogenase (110 U/ml) was then added, the solution mixed and incubated for 5 min at 25°C, and the absorbance ($A_2$) measured. An aliquot (20 μl) of phosphoglucose isomerase (1,000 U/ml) was then added, the solution mixed and incubated for 10 min at 25°C, and the absorbance ($A_3$) measured. Finally, an aliquot (20 μl) of a mixture of galactose dehydrogenase (200 U/ml) and galactose mutarotase (4.1 mg/ml) was added and the solution mixed and incubated for 10 min at 25°C and the absorbance ($A_4$) measured. The difference ($A_2 - A_1$) for both blank (see Figure 4) and sample was determined and the absorbance difference of the blank was subtracted from the absorbance difference of the sample, thereby obtaining $\Delta A_{\text{glucose}}$. The absorbance difference ($A_3 - A_2$) for both blank and sample was determined and the absorbance difference of the blank was subtracted from the absorbance difference of

**FIGURE 4** Procedure for the sequential measurement of glucose, fructose, and galactose in a spectrophotometer cuvette

| Pipette into cuvettes | Blank | Sample |
|---|---|---|
| sample in acetic acid (pH ~ 3.0)<br>solution 4 (sucrase + β-galactosidase) | -<br>- | 0.10 ml<br>0.10 ml |
| Ensure that all of the solutions are delivered to the bottom of the cuvette. Mix the contents by gentle swirling, cap the cuvettes, and incubate them at ~ 25°C for 20 min.<br>**Then add:** | | |
| distilled water (at ~ 25°C)<br>solution 1 (buffer)<br>solution 2 ($NADP^+$/ATP) | 2.20 ml<br>0.10 ml<br>0.10 ml | 2.00 ml<br>0.10 ml<br>0.10 ml |
| Mix*, read the absorbances of the solutions ($A_1$) after approx. 3 min and start the reactions by addition of: | | |
| suspension 5 (HK/G6P-DH) | 0.02 ml | 0.02 ml |
| Mix* and read the absorbances of the solutions ($A_2$) at the end of the reaction (approx. 5 min). If the reaction has not stopped after 5 min, continue to read the absorbances at 2 min intervals until the absorbances remain the same over 2 min**.<br>**Then add:** | | |
| suspension 6 (PGI) | 0.02 ml | 0.02 ml |
| Mix* and read the absorbances of the solutions ($A_3$) at the end of the reaction (approx. 10 min).<br>**Then add:** | | |
| suspension 7 (GalDH/GalM) | 0.02 ml | 0.02 ml |
| Mix*, read the absorbances of the solutions ($A_4$) at the end of the reaction (approx. 10 min). | | |

the sample, thereby obtaining $\Delta A_{\text{fructose}}$. The absorbance difference ($A_4 - A_3$) for both blank and sample was determined and the absorbance difference of the blank was subtracted from the absorbance difference of the sample, thereby obtaining $\Delta A_{\text{galactose}}$.

The concentration of glucose, fructose, and galactose (g/L) was calculated as follows:

$$c = \frac{V \times \text{MW}}{\varepsilon \times d \times v} \times \Delta A \times D.$$

where $V$ = final volume (ml); MW = molecular weight of glucose, fructose, or galactose (g/mol); $\varepsilon$ = extinction coefficient of NADPH at 340 nm = 6,300 ($\text{L} \times \text{mol}^{-1} \times \text{cm}^{-1}$); $d$ = light path (cm); $v$ = sample volume (ml); $D$ = dilution factor (26-fold).

It follows for glucose (g/L):

$$c = \frac{2.42 \times 180.16}{6300 \times 1 \times 0.1} \times \Delta A_{\text{glucose}} \times 26$$
$$= 17.993 \times \Delta A_{\text{glucose}}$$

For fructose (g/L):

$$c = \frac{2.44 \times 180.16}{6300 \times 1 \times 0.1} \times \Delta A_{\text{fructose}} \times 26$$
$$= 18.124 \times \Delta A_{\text{fructose}}$$

For galactose (g/L):

$$c = \frac{2.46 \times 180.16}{6300 \times 1 \times 0.1} \times \Delta A_{\text{galactose}} \times 26$$
$$= 18.290 \times \Delta A_{\text{galactose}}$$

When analyzing solid and semisolid samples that are weighed out for sample preparation, the content (g/100 g) is calculated from the amount weighed as follows:

$$\text{Content of D} - \text{glucose (g/100g)}:$$
$$= c_{\text{D-glucose}}\,(\text{g/L}) \times \text{EV}/1000 \times 1/W \times 100$$

$$\text{Content of D} - \text{fructose (g/100g)}:$$
$$= c_{\text{D-fructose}}\,(\text{g/L}) \times \text{EV}/1000 \times 1/W \times 100$$

$$\text{Content of D-galactose (g/100g)}:$$
$$= c_{\text{D-galactose}}\,(\text{g/L}) \times \text{EV}/1000 \times 1/W \times 100$$

where $c_{\text{D- glucose}}$ (g/L)= concentration of D- glucose per liter of undiluted extraction solution, $c_{\text{D-fructose}}$ (g/L) = concentration of D-fructose per liter of undiluted extraction solution, $c_{\text{D-galactose}}$ (g/L) = concentration of D-galactose per liter of undiluted extraction solution, EV = volume of solution used in the initial extraction (20.5), EV/1,000 = adjustment from g/L of undiluted extraction solution to g/volume of extraction solution actually used, $W$ = weight of sample analyzed in g, and 100 = conversion of results to g/100 g.

$$\text{Available carbohydraytes (g/100g)}$$
$$= \text{D-glucose (g/100g)} + \text{D-fructose (g/100g)}$$
$$+ D\text{-galactose (g/100g)}$$

Calculations are simplified using a Megazyme MegaCalc™ Excel®-based calculator (AVCHO) in Supporting Information.

### 2.8 | Hydrolysis of sucrose, Raftilose® and Raftaline® (FOS) by invertase (β-fructofuranosidase) and sucrase

Aliquots of sucrose, Raftaline® or Raftilose® (1.0 ml, 10 mg/ml), in distilled water were incubated with either invertase (1.0 ml, 200 U on sucrose) in 100 mM sodium acetate buffer (pH 4.5) or sucrase (1.0 ml, 40 U on sucrose) in 100 mM sodium maleate buffer (pH 6.5) containing BSA (0.5 mg/ml) at 30°C. Incubations were terminated at 10, 30, or 60 min by placing the tubes into a boiling water bath for 5 min. A zero time incubation was performed by incubating the enzyme in the boiling water bath for 5 min before adding the sucrose, Raftaline® or Raftilose®. All samples were transferred to microfuge tubes and centrifuged at 8,000 *g* for 3 min. Aliquots of the supernatant solutions were analyzed by HPLC using two TSKgel® G2500PW$_{XL}$ columns, 30 cm × 7.8 mm, connected in series. The columns were operated at 80°C with distilled water mobile phase at 0.5 ml/min. A Bio-Rad Laboratories, cation/anion guard column (cat. no. 125-0118) was employed to deionize the samples.

## 3 | RESULTS AND DISCUSSION

### 3.1 | Measurement of RS (rapid method) and DS

Through the European EURESTA research program, a range of methods for the measurement of RS were developed (Akerberg et al., 1998; Champ, 1992; Champ et al., 1999; Englyst et al., 1992; Faisant et al., 1995; Goni et al., 1996; Muir & O'Dea, 1992), most involved a ~16-hr incubation with pancreatic α-amylase (PAA) with shaking at 37°C. AOAC Method 2002.02, which combines many of the attributes of these methods, was subjected to an interlaboratory evaluation involving 39 laboratories worldwide. The incubation conditions employed in AOAC Method 2009.01 (the integrated TDF method) are based on those used in AOAC Method 2002.02; however, the scale of the assay was increased 10-fold to ensure that sufficient residue was recovered for accurate gravimetric measurement. A major criticism of AOAC Method 2009.01 was that the incubation time with PAA/AMG of 16 hr is not physiologically relevant. In response, a modified method was developed involving an incubation time with PAA/AMG of 4 hr. The concentrations of both PAA and AMG were increased to ensure that RS values obtained with this method (the rapid integrated TDF method) were in line with those obtained with AOAC Methods 2002.02 and 2009.01 and ileostomy studies. This method was successfully evaluated under the auspices of AOAC International and ICC to become AOAC Method 2017.16 and ICC Method 185. In the current work, these assay modifications were applied to the original resistant starch method (AOAC Method 2002.02) in developing a rapid RS method. Incubations are performed in leak-proof, disposable polypropylene tubes, and sample amount (~100 mg) and buffer volume (4 ml) are one-tenth of that employed in AOAC Method 2017.16. Enzyme concentrations (PAA 0.4 KU/4 ml; AMG 0.17 KU/4 ml; Table 1), buffer pH (6.0), incubation time (4 hr), and incubation temperature (37°C) are the same as that employed in AOAC Method 2017.16. Incubations were performed in a shaking water bath (200 linear strokes per minute) with tubes aligned in the direction of shaking to ensure complete sample suspension during the period of incubation (Figure 1). Resistant starch values obtained for a range of samples using AOAC Method 2002.02 and the new rapid RS method are shown in Table 2. Very similar values were obtained for a broad range of samples, but a slightly higher value was obtained for the native, high amylose maize starch, Hylon VII, in line with results obtained with AOAC Method 2017.16 for dietary fiber. The repeatability of the rapid RS method was determined by analyzing seven samples in duplicate over 4 days, and the results are shown in Table 3. Interday repeatability values ranged from 2.17% to 4.84% across a broad range of resistant starch levels, in line with values obtained in previous studies (McCleary et al., 2002) where repeatability values ranged from 1.9% to 3.0%. The very high interday repeatability standard deviation for wheat starch relates to the very low level of RS in this sample. These results demonstrate that the rapid RS method is as repeatable as AOAC Method 2002.02 and that very similar values are obtained for most samples. The significant advantage is that the incubation time is reduced to

**TABLE 2** A comparison of the values obtained for resistant starch content of a range of samples using AOAC Method 2002.02, the rapid resistant starch (RS) method, and RS measured as part of the digestible starch/resistant starch procedure described in this study

| | AOAC 2002.02 | Rapid RS (100 mg samples) | Digestible starch procedure[a] |
|---|---|---|---|
| Regular maize starch (Lot 60401) | 1.0 | 1.2 | 1.6 |
| High amylose maize starch (Lot 60107) | 43.0 | 47.3 | 40.6 |
| Hi Maize 1043® (Lot 02161) | 45.7 | 45.0 | 44.5 |
| Hylon VII® (Ref 98GH8401) | 48.6 | 52.3 | 48.7 |
| Wheat starch (Sigma Lot S512L) | 0.4 | 0.3 | 0.2 |
| Novelose 330® | 42.0 | 37.5 | 37.0 |
| Novelose 240® | 42.9 | 42.6 | 43.1 |
| Crystalline® | 40.9 | 36.7 | 37.6 |
| Native potato starch (Avebe) | 63.4 | 63.9 | 30.8 |
| Potato amylose | 35.6 | 35.3 | 32.9 |
| Actistar® | 49.2 | 49.3 | 47.0 |
| Heinz® baked beans (freeze-dried) | 3.6 | 3.8 | 4.3 |
| Brennans® whole meal bread | 0.9 | 0.8 | 0.7 |
| Canned bachelors butter beans | 3.1 | 3.3 | 3.1 |
| Kellogg's® cornflakes | 2.2 | 2.1 | 1.9 |
| UB express boiled rice | 2.4 | 2.4 | 2.3 |
| Ryvita® dark rye crackers | 1.7 | 1.9 | 1.9 |

[a]In this procedure, an aliquot (4 ml) of the stirred reaction suspension was removed and added to 4 ml of ethanol and RS recovered and washed as in the rapid RS method, hydrolyzed, and analyzed.

the physiologically relevant time of 4 hr, and with this reduced time, there is no requirement for sodium azide preservative in the incubation buffer.

## 3.2 | Measurement of phosphate cross-linked starch (RS4)

It is well known that the resistant starch component of Fibersym® ($RS_4$) is not quantitatively measured under the conditions described for the dissolution and hydrolysis of other resistant starch fractions. $RS_4$ does not dissolve in DMSO at ~100°C and is only partially soluble in 2 M KOH or 1.7 M NaOH at 0–4°C. Shukri et al. (2015) and Shi et al. (2019) have described two quite protracted methods for the dissolution and measurement of $RS_4$.

In the current studies, a range of solvents and incubation conditions were evaluated. Samples were suspended in either 100 mM sodium acetate buffer (pH 4.5) plus $CaCl_2$ at 100°C, 1.7 M NaOH (at 4 or 50°C), or 2 M KOH (at 4°C, as in the rapid RS method). Following pH adjustment of the alkaline solutions to ~pH 5.3, the solutions were incubated with thermostable α-amylase at 100°C followed by AMG at 50°C. In other options, the incubation with thermostable α-amylase at 100°C was deleted (as per the rapid RS method). In all cases, volumes were adjusted to 100 ml and samples were removed for the determination of glucose. In Table 4, the starch values obtained for the RS fraction of Fibersym® using either the Shukri et al. (2015), Shi et al. (2019), or the current 50°C-NaOH procedure are shown. In all cases, Fibersym® was first incubated with PAA/AMG according to the rapid RS method, and then, the residues were dissolved and hydrolyzed according to Shukri et al. (2015), Shi et al. (2019), or the 50°C-NaOH procedure. For Fibersym®, the DS fraction was ~33% *w*/*w* (as is) (average of all samples) and the RS fraction for all three methods was very similar at ~39.5%–42.0% (as is). On direct analysis of the total starch content of Fibersym® with the Shukri et al. (2015) method, a value of 81.3% *w*/*w* (dwb) was obtained, and the Shi method gave ~82.3% *w*/*w* (dwb), while for the 50°C-NaOH procedure a value of ~84.0% *w*/*w* (dwb) was obtained. In no case, values were higher than 84.0% (dwb) obtained. Total starch values obtained for wheat starch using the 50°C-NaOH procedure were ~94.9% *w*/*w* (dwb) and that for Hylon VII were ~95.2% *w*/*w* (dwb) consistent with values obtained with other procedures. In an attempt to further understand the key steps involved in the hydrolysis of Fibersym®, the effect of temperature of incubation in the presence or absence of 1.7 M NaOH, the role of thermostable α-amylase, and the time of incubation with AMG were studied and the results are summarized in Table 5. In each case, DS was hydrolyzed and removed according to the rapid RS method and an average value of 32.8% *w*/*w* (as is basis) was obtained. Values obtained for the RS fraction varied significantly. Under the incubation conditions

**TABLE 3** Repeatability study of the rapid resistant starch (RS) method for the measurement of RS in a range of food materials

| Sample | Resistant starch, % (*w/w*)[a], mean[b] ± 2 *SD*, (%RSD$_r$) | | | | Interday mean, ±2 *SD*, (%RSD$_r$) |
|---|---|---|---|---|---|
| | Day 1 | Day 2 | Day 3 | Day 4 | |
| Heinz® beans [InterLab 13.08.13] | 3.4 ± 0 | 3.7 ± 0 | 3.6 ± 0.2 | 3.7 ± 0 | 3.6 ± 0.3 |
| | 0.04 | 0.44 | 3.10 | 0.63 | 3.91 |
| Starch wheat, unmodified, Sigma S5127, Lot 0490048 | 0.3 ± 0 | 0.4 ± 0.1 | 0.3 ± 0 | 0.3 ± 0 | 0.3 ± 0.1 |
| | 2.21 | 12.47 | 2.24 | 2.15 | 18.01 |
| Kellogg's® corn flakes 20.12.10 | 2 ± 0.1 | 2.2 ± 0.1 | 2.1 ± 0 | 2.1 ± 0 | 2.1 ± 0.2 |
| | 1.61 | 1.82 | 0.54 | 0.23 | 3.78 |
| Tinned chick peas 20/7/11 | 4.3 ± 0.1 | 4.7 ± 0 | 4.6 ± 0.1 | 4.6 ± 0.1 | 4.5 ± 0.3 |
| | 1.28 | 0.39 | 1.56 | 1.26 | 3.40 |
| Semigreen banana | 18.6 ± 0.3 | 18.7 ± 0.6 | 20 ± 1.1 | 20.6 ± 0.4 | 19.5 ± 1.9 |
| | 0.81 | 1.72 | 2.65 | 0.95 | 4.84 |
| Native potato starch Sigma S-4851 Lot 49H04211 | 70 ± 0.5 | 69 ± 1.3 | 71.6 ± 0.5 | 72.7 ± 0.2 | 70.8 ± 3.1 |
| | 0.36 | 0.91 | 0.32 | 0.11 | 2.21 |
| Hylon VII® Lot 60901 | 47.3 ± 1.6 | 48.4 ± 0.5 | 49.5 ± 0.3 | 49.2 ± 2.1 | 48.6 ± 2.1 |
| | 1.69 | 0.50 | 0.30 | 2.17 | 2.17 |
| ActiStar® (before purification) | 46.7 ± 0.6 | 48.6 ± 0.1 | 49.7 ± 0.9 | 51.9 ± 0 | 49.2 ± 4 |
| | 0.69 | 0.10 | 0.89 | 0.03 | 4.07 |

Abbreviations: %RSDr, repeatability standard deviation; *SD*, standard deviation.

[a]All results are presented as starch on an "as is" basis.

[b]On each day, samples of each material were analyzed in duplicate.

**TABLE 4** A comparison of methods employed to measure total starch in Fibersym® and other starches, either directly, or as the combined values of digestible starch and resistant starch

| Sample | Method | Digestible starch (as is) | Resistant starch (as is) | Total starch (as is) | Total starch (dwb) |
|---|---|---|---|---|---|
| Fibersym® | Shukri et al. (2015)[a] | — | — | 72.4 ± 0.4 | 81.3 ± 0.7 |
| | Shukri et al. (2015)[b] | 32.8 ± 0.2 | 39.5 ± 0.1 | 72.3 ± 0.1 | 81.1 ± 0.1 |
| | Shi et al. (2019)[a] | — | — | 73.3 ± 0.2 | 82.3 ± 0.2 |
| | Shi et al. (2019)[b] | 33.0 ± 0.1 | 39.9 ± 0.1 | 72.9 ± 0.1 | 81.8 ± 0.1 |
| | NaOH 15 min at 50°C[a] | — | — | 73.8 ± 0.7 | 82.8 ± 0.8 |
| | NaOH 15 min at 50°C[b] | 32.9 ± 0.2 | 42.0 ± 0.2 | 74.9 ± 0.2 | 84.0 ± 0.3 |
| | NaOH 30 min at 4°C[a] | — | — | 69.7 ± 0.4 | 78.2 ± 0.4 |
| | NaOH 30 min at 4°C[b] | 33.0 ± 0.1 | 37.1 ± 0.9 | 70.1 ± 0.8 | 78.7 ± 0.8 |
| Hylon VII® | Shukri et al. (2015)[a] | — | — | 76.1 ± 0.3 | 87.2 ± 0.3 |
| | Shukri et al. (2015)[b] | 38.7 ± 0.9 | 38.2 ± 2.0 | 76.9 ± 1.5 | 88.1 ± 1.6 |
| | Shi et al. (2019)[a] | — | — | 75.0 ± 0.2 | 86.0 ± 0.2 |
| | Shi et al. (2019)[b] | 38.6 ± 0.5 | 40.3 ± 0.7 | 78.9 ± 0.6 | 90.5 ± 0.7 |
| | NaOH 15 min at 50°C[a] | — | — | 83.0 ± 0.3 | 95.2 ± 0.4 |
| | NaOH 15 min at 50°C[b] | 38.6 ± 0.5 | 43.1 ± 0.6 | 81.7 ± 0.5 | 93.7 ± 0.6 |
| | NaOH 30 min at 4°C[a] | — | — | 83.8 ± 0.6 | 96.2 ± 0.7 |
| | NaOH, 30 min at 4°C[b] | 38.6 ± 0.1 | 43.3 ± 0.6 | 81.9 ± 0.5 | 93.9 ± 0.6 |
| Wheat starch | Shukri et al. (2015)[a] | — | — | 82.3 ± 0.1 | 93.0 ± 0.1 |
| | Shi et al. (2019)[a] | — | — | 78.1 ± 1.2 | 88.2 ± 1.3 |
| | NaOH 15 min at 50°C[a] | — | — | 84.0 ± 1.2 | 94.9 ± 1.3 |
| | NaOH 15 min at 50°C[b] | 84.7 ± 0.7 | 0.2 ± 0.1 | 85.2 ± 0.7 | 96.3 ± 0.8 |
| | NaOH 15 min at 4°C[a] | — | — | 85.3/83.7 | 95.5/93.7 |

*Note:* The Shukri et al. (2015) and Shi et al. (2019) methods were performed as described by the authors except that the initial incubation with PAA/AMG was for 4 hr according to AOAC Method 2017.16. The NaOH/50°C procedure was performed as described in this study.

[a]Total starch determined directly.

[b]Total starch determined as the combined values of digestible and resistant starch.

**TABLE 5** The effect of digestion and enzyme treatments conditions on the measurement of the starch content of Fibersym®

| Samples | NaOH (15 min) Temperature of incubation | α-amylase (280 U, 100°C) Incubation time, min | Incubation time with 300 U AMG (min) | Digestible starch g/100 g "as is"[a] | Resistant starch (g/100 g) "as is" | Total starch (g/100 g) "as is" | Total starch (g/100 g) "dwb" |
|---|---|---|---|---|---|---|---|
| A | Not included | 15 | 30 | 32.8 ± 0.4 | 40.0 ± 0.6 | 72.8 ± 0.5 | 81.7 ± 0.5 |
| B | Not included | 30 | 30 | 32.8 ± 0.4 | 40.5 ± 0.6 | 73.3 ± 0.5 | 82.3 ± 0.5 |
| C | Not included | 60 | 30 | 32.8 ± 0.4 | 40.2 ± 0.5 | 73.0 ± 0.5 | 81.9 ± 0.5 |
| D | 4°C | 0 | 30 | 32.8 ± 0.4 | 1.6 ± 0.2 | 34.4 ± 0.3 | 38.6 ± 0.4 |
| E | 4°C | 30 | 30 | 32.8 ± 0.4 | 39.9 ± 1.0 | 72.7 ± 0.8 | 81.6 ± 0.9 |
| F | 50°C | 0 | 30 | 32.8 ± 0.4 | 5.4 ± 0.6 | 37.2 ± 0.5 | 41.7 ± 0.6 |
| G | 50°C | 30 | 30 | 32.8 ± 0.4 | 41.2 ± 0.5 | 74.0 ± 0.5 | 83.1 ± 0.5 |
| H | 50°C | 30 | 60 | 32.8 ± 0.4 | 41.5 ± 0.6 | 74.3 ± 0.5 | 83.4 ± 0.6 |

*Note:* A. The RS containing residue was suspended in 8 ml of 100 mM sodium acetate buffer (pH 5.0) and incubated with thermostable α-amylase for 15 min and with AMG as shown in the table for 15 min.

B. Incubations performed as for example A, but incubation with thermostable α-amylase for 30 min.

C. Incubations performed as for example A, but incubation with thermostable α-amylase for 60 min.

D. The RS containing residue was suspended in NaOH at 4°C and stirred for 15 min. The solution was then neutralized with acetate buffer and AMG (330 U) was added and incubated at 50°C for 30 min.

E. The RS containing residue was suspended in NaOH at 4°C and stirred for 15 min. The solution was then neutralized with acetate buffer; α-amylase (280 U) was added and incubated at 100°C for 30 min. Temperature was lowered to 50°C, and AMG (330 U) was added and incubated for 30 min.

F. The RS containing residue was suspended in NaOH at 50°C according to the rapid RS method. The solution was then neutralized with acetate buffer, and AMG (330 U) was added and incubated at 50°C for 30 min.

G. The RS containing residue was suspended in NaOH at 50°C according to the rapid RS method. The solution was then neutralized with acetate buffer, and α-amylase (280 U) was added and incubated at 100°C for 30 min. Temperature was lowered to 50°C, and AMG (330 U) was added and incubated for 30 min.

H. Incubations were the same as for "G", except that the incubation with AMG was for 60 min.

[a]The value shown is an average of the determinations for all samples.

employed in the standard rapid RS method (1.7 M NaOH at 4°C) (Example D), little of the RS in Fibersym® was hydrolyzed (~1.6% *w*/*w*). Interestingly, very little of the resistant fraction was hydrolyzed (~5.4% *w*/*w*) even if the NaOH incubation was performed at 50°C (Example F). In all cases where thermostable α-amylase was employed, the highest starch values were obtained. Similar values (~84% *w*/*w*) were obtained on incubation with thermostable α-amylase, whether (examples G and H) or not (examples A–C) the Fibersym® was pre-treated with 1.7 NaOH at 50°C for 15 min. Increasing the incubation time with AMG from 30 to 60 min (examples G and H) gave no increase in the determined values. Increasing the incubation time with 1.7 M NaOH at 50°C from 15 min to either 30, 60, or 120 min (values not shown) gave no change in the determined starch values for the RS fraction of Fibersym®. In no case did the combined value of the digestible starch plus starch from the residue (RS) fraction for Fibersym® (measured as glucose with GOPOD reagent) exceed 84% *w*/*w* (dwb). The starch values determined using either DMSO or NaOH to dissolve Fibersym® are compared in Table 6. With the DMSO format (AOAC Method 996.11), the starch value obtained (66.0%) is approximately 80% of that obtained by the Shukri et al. (2015) procedure (Table 4), consistent with the reports by these authors.

In an attempt to try to understand why quantitative recovery of Fibersym® as glucose in starch-type assays is not achieved, the digested samples (total starch, digestible starch [DS], and

**TABLE 6** Effect of dissolution solvent and temperature of dissolution on measurement of the starch content of Fibersym® and Hylon VII

| | Incubation with solvent | | Incubation time with α-amylase | Total starch, % *w*/*w* dwb | |
|---|---|---|---|---|---|
| Solvent | Time | Temperature | Time at 100°C | Fibersym® | Hylon VII |
| DMSO (2 ml) | 5 min | 100°C | 6 min in MOPS (pH 6.5) | 66.0 ± 0.8 | 94.5 ± 0.9 |
| KOH (2 ml, 2 M) | 20 min | 4°C | 30 min in acetate (pH 5) | 81.3 ± 0.4 | 95.1 ± 1.5 |
| NaOH (2 ml, 1.7 M) | 15 min | 50°C | 30 min in acetate (pH 5) | 83.7 ± 0.2 | 95.6 ± 0.6 |
| Sodium acetate buffer (10 ml, 100 mM) | — | 100°C | 30 min in acetate (pH 5) | 80.5 ± 0.1 | 87.6 ± 0.5 |

digested resistant starch [DRS] fractions) were analyzed for both total carbohydrate by the phenol-sulfuric acid procedure and glucose by the GOPOD method. For reference, Hylon VII® was analyzed concurrently and the determined glucose was ~98% of the total carbohydrate value. For Fibersym®, glucose was ~92% of the total carbohydrate value, clearly demonstrating that the hydrolyzate still contained ~8% of chemically modified glucose/gluco-oligosaccharides that is resistant to hydrolysis to glucose by AMG and thus not measured by the glucose-specific GOPOD reagent. To further identify the nature of the resistant fractions, Fibersym® was hydrolyzed with PAA/AMG according to the rapid resistant starch procedure (for 4 hr at 37°C). The DS fractions from 20 incubations were pooled, concentrated, and chromatographed on Bio-Gel P-2. The residues fractions in the PAA/AMG incubations were also pooled, washed with 50% aqueous ethanol, stirred in 1.7 M NaOH at 50°C for 30 min, neutralized, and hydrolyzed with thermostable α-amylase for 30 min at 100°C followed by AMG for 30 min at 50°C. Under these conditions, the residue was essentially completely solubilized. The DRS fraction was also concentrated and chromatographed on Bio-Gel P-2. Fractions were collected and analyzed for carbohydrate (Figure 5) using the phenol-sulfuric acid procedure (DuBois et al., 1956). The DS fraction consisted of 89% monosaccharide (shown to be exclusively glucose using GOPOD reagent), 4% disaccharide, 2% trisaccharide, and 5% higher DP oligosaccharides. The di- and trisaccharides were completely hydrolyzed to glucose by AMG, showing that they were maltose and maltotriose that escaped hydrolysis by AMG in the initial incubation with PAA/AMG. The higher DP fraction was hydrolyzed to an extent of <5% by AMG. Thus, the digestible starch fraction contained ~5% of oligosaccharides resistant to digestion by AMG, and this represents 5 × 32.8/100% (i.e., 1.6%) of the total Fibersym® sample. The DRS fraction consisted of 87% monosaccharide (shown to be exclusively glucose using GOPOD reagent), ~7% of disaccharides, 1% trisaccharide, and ~5% higher DP oligosaccharides. The di- and trisaccharide fractions (~8%) were resistant to hydrolysis by AMG, and the higher DP fraction (~ 5%) was hydrolyzed to an extent of <5% by AMG (i.e., 4.8% resistant oligosaccharides). Thus, the combined resistant fractions in DRS were ~13% of the total hydrolyzate. From Table 5, Example "B," the DS fraction is 32.8% "as is" and the resistant starch fraction is 42.8% "as is" determined as glucose. In allowing for the ratio of determined glucose to total carbohydrate contents (phenol-sulfuric) of these two fractions, the total recovery of the sample is 32.8 × 100/95 (DS fraction) + 42.8 × 100/87 (DRS fraction) = 34.5 + 49.2, or 83.7% "as is" (i.e., 93.7% "dwb"). The resistant starch fraction is ~55% "dwb" (from the DRS fraction) plus ~2% (from the DS fraction), that is, 57% dwb, consistent with values obtained by gravimetry in AOAC Method 2017.16.

Hydrolysis of starch by PAA/AMG mixtures is critically dependent on both the concentrations of the enzymes used and the time of incubation. In AOAC Method 2002.02, 0.2 KU of PAA and 0.014 KU of AMG are employed per assay (in 4.0 ml of buffer) and incubations are performed at 37°C for 16 hr. This incubation time was chosen on the basis of previously published work (Akerberg et al., 1998; Champ, 1992; Champ et al., 1999; Faisant et al., 1995; Goni et al., 1996; Muir & O'Dea, 1992) and particularly because the RS value obtained for a set of reference samples was in line with results from ileostomy studies (Champ et al., 1999). The time course hydrolysis of a range of starches under these conditions is shown in Figure 6. In their studies on measurement of the RS content of

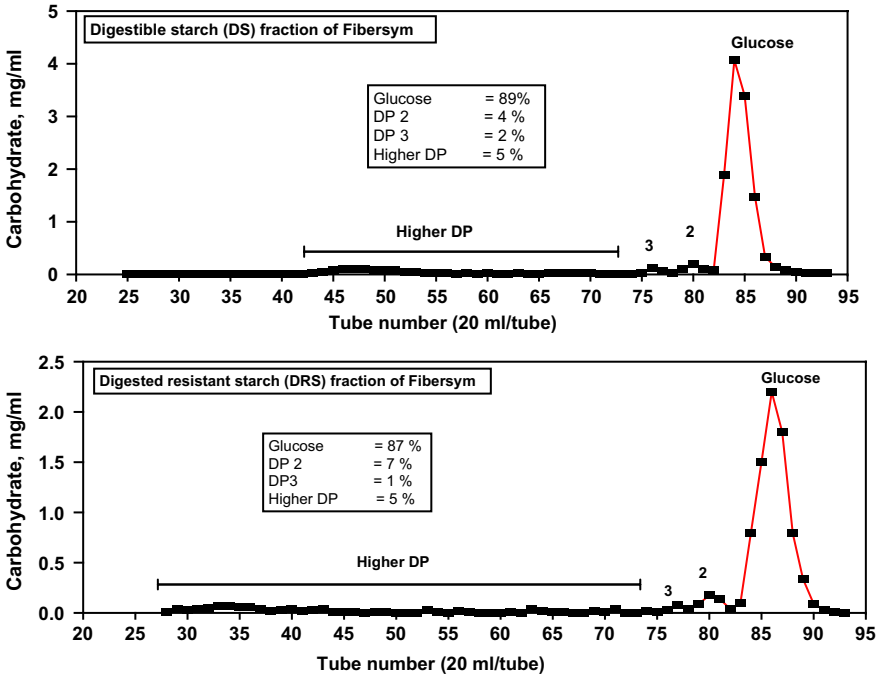


**FIGURE 5** Chromatography on Bio-Gel P-2 of the "digestible starch" and hydrolysed "resistant starch" fractions of Fibersym®. Samples (20 ml, 20, or 30 mg/ml) were applied and eluted with distilled water at 60°C. Fractions (20 ml) were collected and analyzed for carbohydrate using the phenol-sulfuric acid procedure [Color figure can be viewed at wileyonlinelibrary.com]

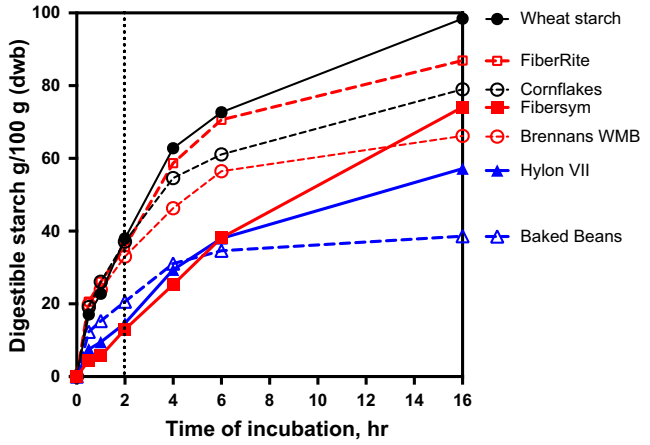


**FIGURE 6** Time course hydrolysis of a range of starches and starch-containing foods by a mixture of pancreatic α-amylase and amyloglucosidase under the incubation conditions of the integrated TDF procedure (AOAC Method 2009.01). Samples (0.5 ml) were removed at various time intervals, the enzymes inactivated, and glucose measured according to Materials and Methods [Color figure can be viewed at wileyonlinelibrary.com]

Fibersym®, both Shukri et al. (2015) and Shi et al. (2019) used the incubation conditions of AOAC Method 2002.02, except that the incubation time with PAA/AMG was reduced from 16 to 2 hr. Not surprisingly, much less of the Fibersym® was hydrolyzed, and thus, much higher RS values were obtained. From Figure 6, it can be seen that under the incubation conditions of AOAC Method 2002.02, Fibersym® is hydrolyzed to an extent of ~70% after 16 hr (i.e., ~30% RS), but only 13% after 2 hr. With an incubation time of 2 hr, even wheat starch has a RS value of ~60% *w/w*, which clearly shows that using the PAA/AMG content employed in AOAC method 2002.02 with this shortened incubation time generates nonsensical results. AOAC Method 2009.01 employs the incubation conditions of AOAC Method 2002.02, and a DF value of ~30% *w/w* was obtained for Fibersym®. This method was recently updated (AOAC Method 2017.16) by reducing the incubation time to 4 hr while increasing the concentrations of both PAA and AMG to ensure that the RS values obtained with a set of reference samples still aligned with ileostomy data. Under these conditions, a RS value of ~60% *w/w* was obtained for Fibersym®, still significantly less than the value of ~86% *w/w* (dwb) reported by Maningat, Seib, and Bassi (2013).

## 3.3 | Measurement of digestible starch and resistant starch

The method described here for the measurement of digestible starch and resistant starch is aligned closely to the method of Englyst et al. (1992), with the modification that samples are removed for analysis at 20, 120 min, and also 240 min. Furthermore, the resistant starch content of the sample is measured directly rather than by difference as in the Englyst et al. (1992) procedure. A further difference is that highly purified, standardized enzymes are employed and incubations are performed in tubes that allow sample removal while the incubation is continuing (Figure 3). Incubation conditions are identical to those in AOAC Method 2017.16 except that sample size, buffer volumes, and amounts of PAA and AMG are all reduced twofold. Enzyme concentration, incubation pH and temperature, and stirring conditions are the same. Consistent with the method of Englyst et al. (1992), sample aliquots are removed at 20 min to measure rapidly digested starch (RDS) and 120 min to measure slowly digested starch (SDS). However, a third sample is removed at 240 min (4 hr) to measure total digestible starch (TDS); [see "Measurement of digestible (RDS, SDS, TDS) and resistant starch"—Methods]. This time is in line with the reported time of residence of food in the human small intestine (Camalilleri et al., 2010; Deiteren et al., 2010; Geboes et al., 2003; Geypens et al., 1999; Miller et al., 1997; Sadik et al., 2003; Stotzer & Abrahamsson, 2010; Zarate et al., 2010). After 240 min (4 hr) of incubation, a sample (4 ml) is removed to measure resistant starch. The sample is added to 4 ml of ethanol, and all further washing, dissolution, and hydrolysis steps are the same as those employed in the rapid RS method. The repeatability of this method for the measurement of RDS, SDS, TDS, and RS is shown in Tables 7–10. Seven samples with a broad range of digestible and resistant starch values were analyzed, and excellent repeatability was obtained for each value for all of the samples. Resistant starch values determined for a range of samples with the digestible/resistant starch method are compared to values obtained with AOAC Method 2002.02 and the rapid RS method in Table 2. Similar values were obtained with each of the procedures with the clear exception of native potato starch, which is known to be a fragile starch and readily damaged under incubation conditions that involve stirring (McCleary & Monaghan, 2002).

## 3.4 | Measurement of available carbohydrates

Available carbohydrates have been defined as the sum of sugars (glucose, fructose, galactose, sucrose, maltose, lactose, and oligosaccharides) and complex carbohydrates ("malto" dextrins, starch, and glycogen; Anon, 2003). Historically, these have been measured individually by a combination of enzymatic and HPLC procedures and the values pooled. The method described here involves the complete and specific hydrolysis of each carbohydrate to the component monosaccharides, glucose, fructose, and galactose and specific enzymatic measurement of these in a single reaction cuvette. With the increased knowledge of starch hydrolysis in the human small intestine and the recognition of the importance of resistant starch as a component of dietary fiber,

**TABLE 7** Repeatability study on the measurement of "rapidly digested starch" in the digestible starch-resistant starch assay procedure

| Sample | Rapidly digested starch, % (*w/w*)[a], mean[b] ± 2 *SD*, (%RSD$_r$) | | | | Interday mean, ±2 *SD*, (%RSD$_r$) |
|---|---|---|---|---|---|
| | Day 1 | Day 2 | Day 3 | Day 4 | |
| Regular maize starch | 21 ± 1.4 | 19.9 ± 0.4 | 19.2 ± 1.3 | 19.7 ± 0.2 | 19.9 ± 1.6 |
| | 3.29 | 0.91 | 3.32 | 0.46 | 3.95 |
| Hylon VII® | 7.4 ± 0.3 | 6.8 ± 0.5 | 6.5 ± 0.3 | 7 ± 0.4 | 6.9 ± 0.8 |
| | 2.35 | 3.61 | 2.60 | 2.78 | 5.52 |
| UB express boiled rice | 58.9 ± 2.7 | 59.3 ± 3.9 | 57.3 ± 0.7 | 57.2 ± 2.6 | 58.2 ± 2.9 |
| | 2.29 | 3.32 | 0.60 | 2.24 | 2.48 |
| ActiStar® | 21.2 ± 0.2 | 20.6 ± 0.9 | 20.3 ± 0.8 | 22.5 ± 0.7 | 21.2 ± 1.9 |
| | 0.43 | 2.11 | 1.91 | 1.65 | 4.38 |
| Garden peas | 13 ± 0.1 | 12.7 ± 0.3 | 12.8 ± 0 | 12.7 ± 0.1 | 12.8 ± 0.4 |
| | 0.38 | 1.23 | 0.11 | 0.56 | 1.37 |
| All bran | 23.6 ± 0.3 | 24 ± 0.3 | 24.1 ± 0.2 | 23.9 ± 0 | 23.9 ± 0.4 |
| | 0.56 | 0.62 | 0.44 | 0.03 | 0.83 |
| Butter beans | 17.9 ± 0.5 | 18.5 ± 0.2 | 19.3 ± 1.8 | 19.1 ± 1.4 | 18.7 ± 1.4 |
| | 1.46 | 0.63 | 4.75 | 3.55 | 3.81 |

Abbreviations: %RSDr, repeatability standard deviation; *SD*, standard deviation.
[a]All results are presented as starch on an "as is" basis.
[b]On each day, samples of each material were analyzed in duplicate.

**TABLE 8** Repeatability study on the measurement of "slowly digested starch" in the digestible starch-resistant starch assay procedure

| Sample | Slowly digested starch, % (*w/w*)[a], mean[b] ± 2 *SD*, (%RSD$_r$) | | | | Interday mean, ±2 *SD*, (%RSD$_r$) |
|---|---|---|---|---|---|
| | Day 1 | Day 2 | Day 3 | Day 4 | |
| Regular maize starch | 51.1 ± 2.5 | 48.4 ± 1.7 | 49.8 ± 1.2 | 47.3 ± 1.3 | 49.2 ± 3.3 |
| | 2.47 | 1.78 | 1.24 | 1.33 | 3.39 |
| Hylon VII® | 14.3 ± 0.1 | 16.5 ± 0.2 | 16.5 ± 0.5 | 17.3 ± 0.1 | 16.2 ± 2.4 |
| | 0.31 | 0.75 | 1.62 | 0.42 | 7.42 |
| UB express boiled rice | 13.6 ± 3.8 | 12.3 ± 1.4 | 11.7 ± 1.3 | 11.4 ± 0.4 | 12.2 ± 2.4 |
| | 13.85 | 5.61 | 5.43 | 1.69 | 9.77 |
| ActiStar® | 5.3 ± 1.1 | 6 ± 0.2 | 5.7 ± 0 | 7 ± 0.2 | 6 ± 1.4 |
| | 10.44 | 1.78 | 0.22 | 1.62 | 11.63 |
| Garden peas | 2.6 ± 0.3 | 2.7 ± 0.6 | 2.6 ± 0.1 | 2.5 ± 0.1 | 2.6 ± 0.3 |
| | 4.97 | 11.28 | 2.44 | 1.15 | 5.97 |
| All bran | 1.4 ± 1.1 | 1.2 ± 0.7 | 0.7 ± 0.3 | 0.6 ± 0.3 | 1 ± 0.9 |
| | 40.84 | 30.81 | 19.00 | 22.52 | 45.75 |
| Butter beans | 14.1 ± 2 | 13.3 ± 0.4 | 12.7 ± 0.3 | 12 ± 1.8 | 13 ± 2 |
| | 7.21 | 1.62 | 1.35 | 7.44 | 7.55 |

Abbreviations: %RSDr, repeatability standard deviation; *SD*, standard deviation.
[a]All results are presented as starch on an "as is" basis.
[b]On each day, samples of each material were analyzed in duplicate.

it has become important to specifically measure digestible starch, rather than total starch, for the calculation of available carbohydrates. Consequently, the procedure described here for the measurement of total digestible starch (TDS) forms the basis of the current method for available carbohydrates. Samples are incubated with PAA and AMG under the conditions described for the digestible starch/resistant starch procedure. A sample of the incubation solution is removed and added to dilute acetic acid, and this is used in the determination of available carbohydrates. In the incubation with PAA and AMG, maltose, maltodextrins, glycogen, and digestible starch are hydrolyzed to glucose (with trace levels

**TABLE 9** Repeatability study on the measurement of "total digestible starch" in the digestible starch-resistant starch assay procedure

| Sample | Total digested starch, % (*w/w*)[a], mean[b] ± 2 *SD*, (%RSD$_r$) | | | | Interday mean, ±2 *SD*, (%RSD$_r$) |
|---|---|---|---|---|---|
| | Day 1 | Day 2 | Day 3 | Day 4 | |
| Regular maize starch | 82.1 ± 0.4 | 79 ± 0.8 | 79.5 ± 0.4 | 79.8 ± 1.7 | 80.1 ± 2.7 |
| | 0.25 | 0.54 | 0.23 | 1.07 | 1.67 |
| Hylon VII® | 33.2 ± 0.9 | 36.5 ± 1.7 | 35.7 ± 0.2 | 38.2 ± 0.6 | 35.9 ± 3.9 |
| | 1.34 | 2.39 | 0.32 | 0.84 | 5.47 |
| UB express boiled rice | 72.5 ± 1.3 | 70.2 ± 0.9 | 70.8 ± 0.4 | 71.8 ± 0.1 | 71.3 ± 2 |
| | 0.93 | 0.66 | 0.29 | 0.06 | 1.40 |
| ActiStar® | 34.6 ± 0.4 | 34.1 ± 4.1 | 33.1 ± 0.8 | 35.6 ± 2.3 | 34.4 ± 2.7 |
| | 0.55 | 6.05 | 1.21 | 3.21 | 3.89 |
| Garden peas | 16.9 ± 0 | 16.6 ± 0.4 | 16.3 ± 0.5 | 16.4 ± 0.1 | 16.5 ± 0.6 |
| | 0.04 | 1.11 | 1.61 | 0.17 | 1.77 |
| All bran | 24.9 ± 1.5 | 25.2 ± 0.2 | 25.1 ± 0.1 | 25.1 ± 0.2 | 25.1 ± 0.6 |
| | 2.93 | 0.32 | 0.22 | 0.45 | 1.27 |
| Butter beans | 34.4 ± 0.6 | 34.5 ± 0.2 | 34.7 ± 0 | 34.9 ± 1.8 | 34.6 ± 0.8 |
| | 0.87 | 0.28 | 0.05 | 2.54 | 1.20 |

Abbreviations: %RSDr, repeatability standard deviation; *SD*, standard deviation.
[a]All results are presented as starch on an "as is" basis.
[b]On each day, samples of each material were analyzed in duplicate.

of maltose). On subsequent incubation with sucrase, maltase, and β-galactosidase, lactose is hydrolyzed to glucose and galactose, sucrose is specifically hydrolyzed to glucose and fructose, and maltose is hydrolyzed to glucose (Figure 4). Measurement of glucose, fructose, and galactose is shown in Figure 7. Traditionally, sucrose has been hydrolyzed using invertase (β-fructofuranosidase), but this enzyme also acts on fructo-oligosaccharides resulting in overestimation of the sucrose. Hydrolysis of sucrose and Raftilose® (a commercial fructo-oligosaccharide mixture) by invertase and sucrase is shown in Figure 8. Incubation conditions with invertase are in line with those used in commercial sucrose assay kits. Under

**TABLE 10** Repeatability study on the measurement of "resistant starch" in the digestible starch-resistant starch assay procedure

| Sample | Resistant starch, % (*w/w*)[a], mean[b] ± 2 *SD*, (%RSD$_r$) | | | | Interday mean, ±2 *SD*, (%RSD$_r$) |
|---|---|---|---|---|---|
| | Day 1 | Day 2 | Day 3 | Day 4 | |
| Regular maize starch | 1.9 ± 0.1 | 2 ± 0.1 | 1.9 ± 0 | 2 ± 0.1 | 2 ± 0.1 |
| | 1.52 | 2.77 | 0.07 | 2.77 | 2.47 |
| Hylon VII | 48.2 ± 0.1 | 47.7 ± 1.9 | 47.1 ± 0.2 | 46.6 ± 0.6 | 47.4 ± 1.5 |
| | 0.14 | 1.95 | 0.17 | 0.60 | 1.60 |
| UB express boiled rice | 2.5 ± 0.4 | 2.6 ± 0.3 | 2.6 ± 0.2 | 2.4 ± 0.2 | 2.5 ± 0.3 |
| | 8.06 | 5.22 | 4.58 | 3.28 | 6.33 |
| ActiStar | 52 ± 0.1 | 52.3 ± 0.3 | 51 ± 0.1 | 51.8 ± 0.9 | 51.8 ± 1.1 |
| | 0.07 | 0.25 | 0.13 | 0.88 | 1.05 |
| Garden peas | 7.9 ± 0.4 | 7.9 ± 0.3 | 7.6 ± 0.7 | 8 ± 0.6 | 7.9 ± 0.5 |
| | 2.36 | 1.69 | 4.55 | 3.42 | 3.09 |
| All bran | 0.3 ± 0 | 0.3 ± 0 | 0.3 ± 0 | 0.3 ± 0 | 0.3 ± 0 |
| | 4.36 | 0.95 | 0.14 | 3.69 | 3.96 |
| Butter beans | 3.5 ± 0.1 | 3.4 ± 0.1 | 3.5 ± 0.2 | 3.3 ± 0 | 3.4 ± 0.2 |
| | 1.43 | 1.16 | 2.24 | 0.38 | 3.22 |

Abbreviations: %RSDr, repeatability standard deviation; *SD*, standard deviation.
[a]All results are presented as starch on an "as is" basis.
[b]On each day, samples of each material were analyzed in duplicate.

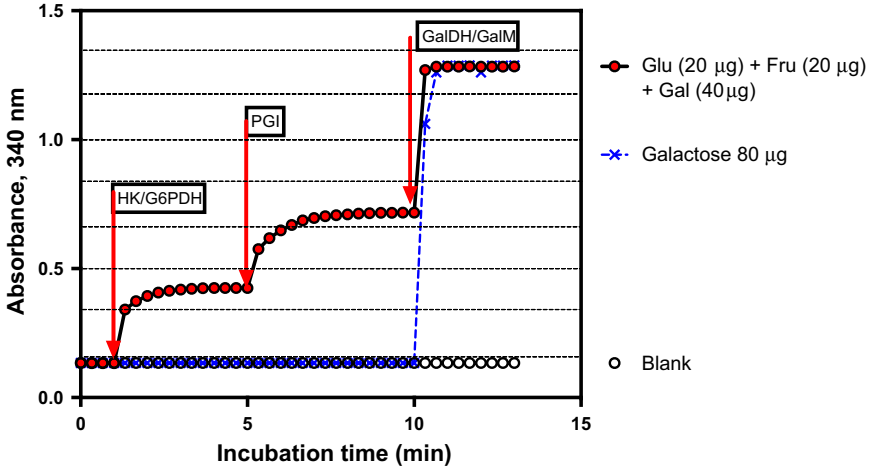


**FIGURE 7** Time course measurement of glucose, fructose, and galactose under the incubation conditions described in Figure 4 [Color figure can be viewed at wileyonlinelibrary.com]

these incubation conditions, sucrase gives complete hydrolysis of sucrose after 60 min (Sucrose + Sucrase/60) but has no action on Raftilose (Raftilose + Sucrase/60) as required in the available carbohydrates method. In contrast, invertase completely hydrolyses sucrose to glucose and fructose within 10 min and also gives near-complete hydrolysis of Raftilose® in the same time (Raftilose + Invertase/10). Clearly, invertase is unsuitable for the specific hydrolysis of sucrose in

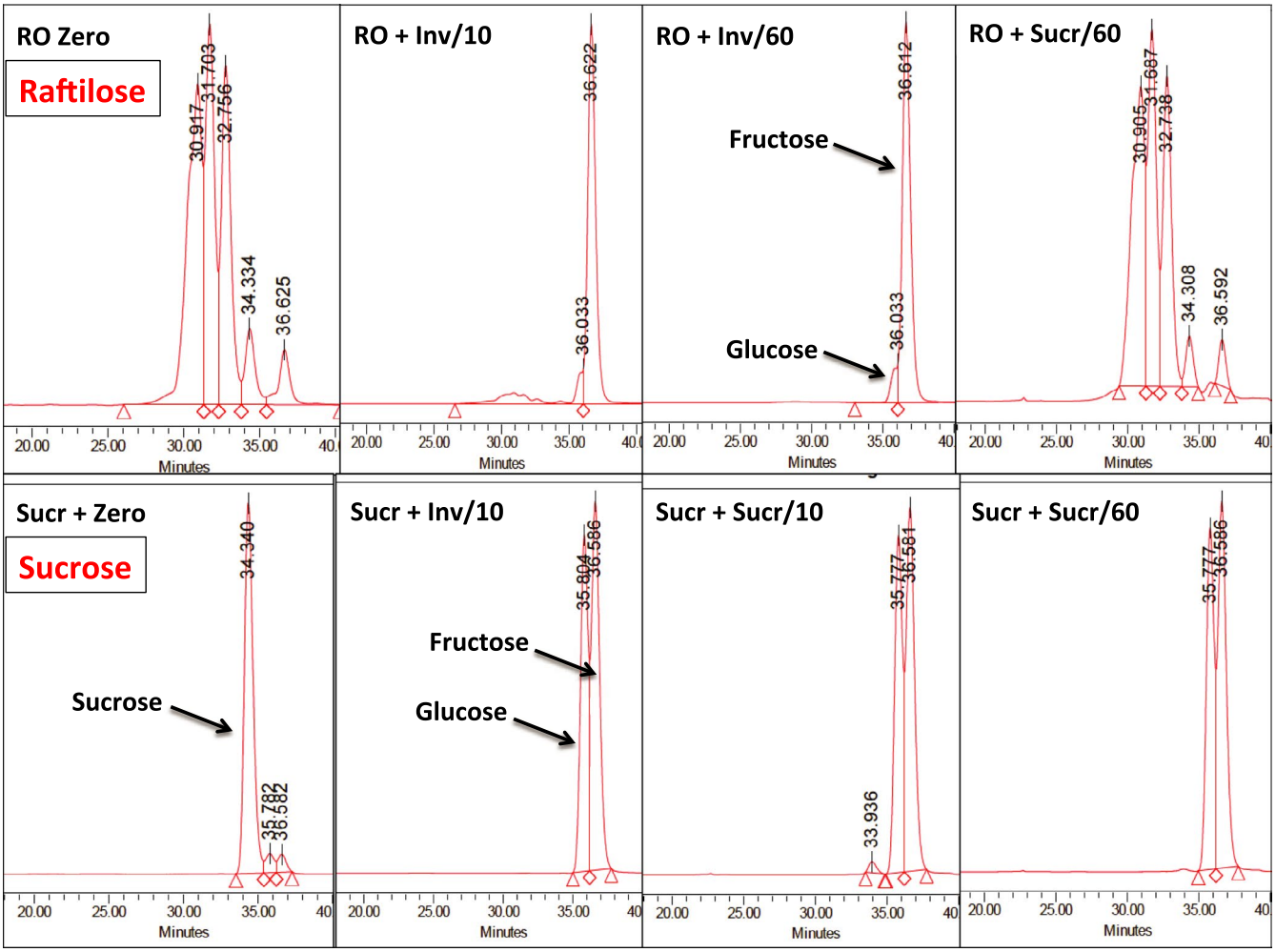


**FIGURE 8** HPLC chromatography on two TSKgel® G2500PWXL columns of the products of hydrolysis of sucrose and Raftilose® by either invertase or sucrase enzymes as described in Materials and Methods [Color figure can be viewed at wileyonlinelibrary.com]

the presence of fructo-oligosaccharides. The β-galactosidase employed here is from *Aspergillus niger* and is known to give complete hydrolysis of galacto-oligosaccharides (GOS) as well as lactose. More specific hydrolysis of lactose can be achieved with β-galactosidase from *Escherichia coli*, but different incubation conditions (pH ~ 7.5) would be required. Since GOS are rarely used in food products other than specific baby formulations, the *A. niger* β-galactosidase was chosen for this methodology. The incubation format employed in the measurement of available carbohydrates is shown in Figure 4. This differs from previously published formats in that galactose is also measured.

The content of glucose, fructose, and available carbohydrates content of a number of food products is shown in Table 11. These products contained no lactose or galactose. In Table 12, the available carbohydrates content of three food products, two of which contain lactose, is shown. Repeatability of the available carbohydrates method has been determined by analyzing eight food products in duplicate over 4 days (Table 13). Interday repeatability is excellent, with $RSD_r$ values ranging from 1.6 to 3.58.

# 4 | CONCLUSIONS

## 4.1 | Digestible and resistant starch

In this paper, we describe simple, reliable, and accurate methods for the measurement of resistant starch (RS), digestible starch (RDS, SDS, and TDS), and available carbohydrates and highlight key considerations in each determination. Accurate hydrolysis of digestible (nonresistant) starch is a critical element of all of these determinations. The incubation conditions employed in each of these assays mimic those used in the rapid integrated total dietary fiber method (AOAC Method 2017.16, ICC Method 185); the ratio of sample weight, buffer volume, and concentration of enzymes are the same. Under incubation conditions designed to measure total dietary fiber (AOAC Method 2017.16), available carbohydrate values can be obtained simply by removing aliquots

**TABLE 11** Available carbohydrate contents[a] of a range of samples ("as is" basis, or freeze-dried as stated)

| Samples | D-glucose (g/100 g) | D-fructose (g/100 g) | Available carbohydrates (g/100 g) |
|---|---|---|---|
| Kellogg's® cornflakes | 76.1 | 2.8 | 78.9 |
| Kellogg's® all bran | 37.9 | 7.1 | 45.0 |
| Weetabix® | 68.1 | 1.1 | 69.2 |
| Kellogg's® Special K® | 69.4 | 4.2 | 73.6 |
| Kellogg's® Frosties® | 67.1 | 13.4 | 80.5 |
| Roma® macaroni pasta | 69.1 | 0.0 | 69.1 |
| Rooster potato (freeze-dried) | 50.9 | 0.8 | 51.7 |
| Sweet potato (freeze-dried) | 46.6 | 17.6 | 64.2 |
| Red onion (freeze-dried) | 28.2 | 24.2 | 52.4 |
| Cauliflower (freeze-dried) | 11.8 | 11.4 | 23.2 |
| Celery (freeze-dried) | 12.1 | 9.5 | 21.6 |
| Broccoli (freeze-dried) | 7.1 | 6.2 | 13.3 |
| Carrot (freeze-dried) | 26.8 | 18.3 | 45.1 |
| Swede (freeze-dried) | 33.8 | 17.4 | 51.2 |
| Red pepper (freeze-dried) | 24.3 | 37.9 | 62.2 |
| Mushroom (freeze-dried) | 3.1 | 0 | 3.1 |
| Ripe banana (freeze-dried) | 39.8 | 29.4 | 69.2 |
| Red kidney beans (dry) | 12.1 | 2.6 | 14.7 |
| Soya bean (dry) | 3.9 | 3.2 | 7.1 |
| Heinz® baked beans (freeze-dried) | 43.2 | 3.5 | 48.7 |
| Ryvita® dark rye crackers | 68.7 | 4.8 | 73.5 |
| Wheat starch | 86.3 | 0.0 | 86.3 |
| Hylon VII® | 33.4 | 0.0 | 33.4 |
| Potato amylose | 59.4 | 0.0 | 59.4 |
| Regular maize starch | 83.7 | 0.0 | 83.7 |

[a]All values are the average of duplicate determinations.

**TABLE 12** Glucose, fructose, galactose, and available carbohydrate contents[a] of three defatted and freeze-dried food samples

| Sample | Glucose (g/100 g) | Fructose (g/100 g) | Galactose (g/100 g) | Available carbohydrates (g/100 g) |
|---|---|---|---|---|
| Chocolate chip cookies | 43.9 | 1.78 | 0.75 | 45.45 |
| Chocolate chip cookies | 43.17 | 1.78 | 0.67 | 45.63 |
| Chocolate peanuts | 11.22 | 2.34 | 6.34 | 19.90 |
| Chocolate peanuts | 11.26 | 2.35 | 6.34 | 19.95 |
| Jam tarts | 57.80 | 10.24 | 0.00 | 68.04 |

[a]All values are the average of duplicate determinations and on a dry weight basis.

**TABLE 13** Repeatability study on the measurement of available carbohydrates (glucose plus fructose) in a range of food products

| Sample | Available carbohydrates, % (*w*/*w*)[a], mean[b] ± 2 *SD*, (%RSD$_r$[c]) | | | | Interday mean, ±2 *SD*, (%RSD$_r$) |
|---|---|---|---|---|---|
| | Day 1 | Day 2 | Day 3 | Day 4 | |
| Wheat starch | 87.3 ± 2.1 | 90.2 ± 2.2 | 88.2 ± 0.6 | 90 ± 1.1 | 88.9 ± 2.8 |
| | 1.21 | 1.21 | 0.34 | 0.61 | 1.60 |
| All bran | 43.2 ± 2.2 | 45.4 ± 0.4 | 43.2 ± 1 | 44.5 ± 0.2 | 44.1 ± 2.2 |
| | 2.55 | 0.40 | 1.20 | 0.27 | 2.53 |
| Sweet potato | 59.6 ± 0.2 | 60.7 ± 1.3 | 58.2 ± 2.1 | 60.4 ± 1 | 59.7 ± 2.3 |
| | 0.14 | 1.10 | 1.80 | 0.81 | 1.92 |
| Ripe banana | 65.4 ± 0.4 | 70 ± 0.3 | 67.1 ± 1 | 66.8 ± 0.6 | 67.3 ± 3.6 |
| | 0.28 | 0.19 | 0.77 | 0.46 | 2.68 |
| Carrot | 53.7 ± 0.9 | 57.4 ± 0.6 | 55.1 ± 1.5 | 55.3 ± 0.3 | 55.4 ± 2.9 |
| | 0.87 | 0.54 | 1.36 | 0.23 | 2.58 |
| Red pepper | 51 ± 0.5 | 55.4 ± 2.5 | 53.8 ± 3.1 | 52.9 ± 1.9 | 53.2 ± 3.8 |
| | 0.49 | 2.25 | 2.92 | 1.83 | 3.58 |
| Ryvita® | 60.6 ± 1.3 | 61.5 ± 1.3 | 61 ± 2.4 | 62.6 ± 0.7 | 61.4 ± 2 |
| | 1.07 | 1.04 | 1.96 | 0.60 | 1.61 |
| Swede | 55 ± 4.3 | 53.6 ± 0.4 | 54.2 ± 1.3 | 54.1 ± 2 | 54.2 ± 2.1 |
| | 3.92 | 0.34 | 1.19 | 1.82 | 1.96 |

*Note:* The repeatability (%RSD$_r$) of the available carbohydrates assay method was assessed using eight milled samples. For each sample, duplicate extractions were processed and applied to the assay on each day across four separate days.

The available carbohydrate content of the samples tested covered a working range of 44.1%–88.9% (*w*/*w*) on a dry weight basis. The repeatability (%RSD$_r$) across this sample data set was excellent, less than or equal to 3.58% for all samples.

[a]All results are presented on a dry weight basis.

[b]On each day samples of each material were analyzed in duplicate.

[c]SD = standard deviation. %RSD$_r$ = repeatability standard deviation.

(e.g., 0.2 ml) from the incubation solutions for available carbohydrate analysis. The digestible starch methodology described here allows a comparison of the rates of digestion of various starches and should be useful in monitoring food processing conditions aimed at producing slowly digested and resistant starch. As detailed here, "available carbohydrates" are determined on samples removed at an incubation time of 4 hr. However, a measure of "glycemic carbohydrates," that is, the carbohydrates which affect the glycemic index, can be obtained by removing samples from the incubation mixture at 20 min. Englyst, Englyst, Hudson, Cole, and Cummings (1999) have shown a good correlation between rapidly available glucose (RAG) and insulin response. RAG is derived from free glucose, maltodextrins, and starch that are digested within 20 min. In the current method, separate measurement of the glucose, fructose, and galactose derived from rapidly digested carbohydrates, together with the known glycemic index for each sugar, makes it possible to determine the glycemic index of the food.

## 4.2 | Phosphate cross-linked starch (RS4)

Digestible starch is the major contributor to available carbohydrates in many foods. Starch has been modified in several

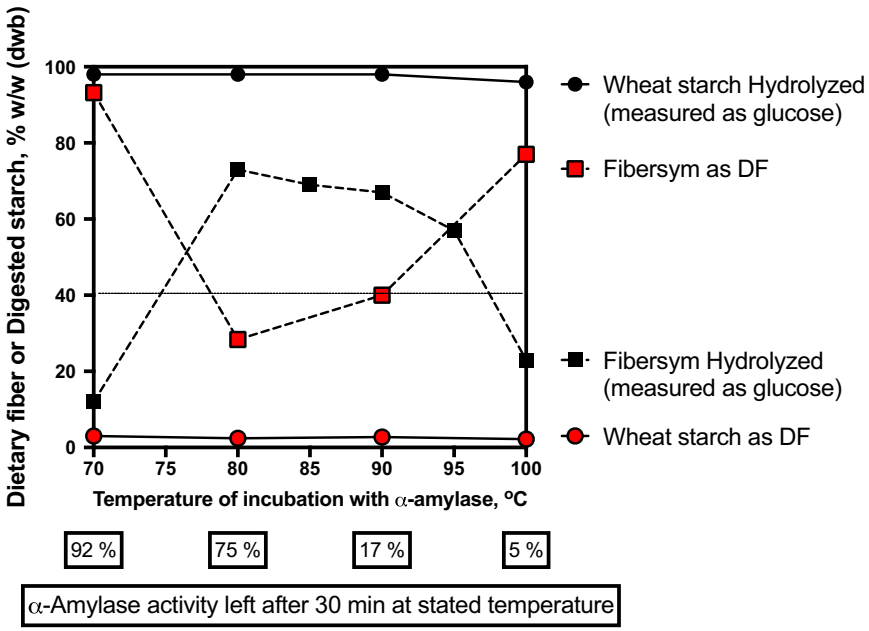


**FIGURE 9** Hydrolysis of wheat starch and Fibersym® under conditions exactly as described for AOAC Method 985.29, but with incubations performed at 70, 80, 90, 95, and 100°C. Incubations with protease and AMG were as described in AOAC Method 985.29. A sample of the incubation solution (10 ml) was removed and immediately filtered. A sample (1 ml) was added to 25 ml of distilled water and mixed. An aliquot (0.1 ml) of this was incubated with 30 U of AMG at 40°C for 15 min and measured for glucose using a glucose oxidase/peroxidase reagent. DS was calculated from glucose value, and the dietary fiber value (RS) was determined by subtracting the DS value from the total sample weight. The α-amylase activity remaining in the incubation mixtures was determined using the Ceralpha® method [Color figure can be viewed at wileyonlinelibrary.com]

ways to reduce digestibility and contribute positively to the dietary fiber content of the food. One of the most widely used, chemically modified starches is phosphate cross-linked starch (e.g., Fibersym®). Measurement of this, either directly or indirectly, is a matter of ongoing debate. Maningat et al. (2013) have reported fiber values for phosphate cross-linked starch (e.g., Fibersym®) in excess of 85% *w/w* (dwb) using the Prosky et al. (1985) dietary fiber method (AOAC Method 985.29/AACC Method 32-05.01). To obtain these values, the incubation step with heat-stable α-amylase must be performed at 98–100°C. At incubation temperature below 98°C, the determined dietary fiber value plummets (Figure 9). For example, at an incubation temperature of 95°C, a DF value of just 43% is obtained and this drops to 28% at 80°C. Clearly, the values obtained are method dependent and the method employed has no relationship to digestion conditions in the human small intestine, and therefore, the Prosky dietary fiber method is simply unsuitable for use with samples containing $RS_4$. AOAC Method 2009.01 was developed as an alternative method for the measurement of total dietary fiber with an attempt to obtain physiologically relevant measurement of RS. However, since an incubation time with PAA/AMG of 16 hr was employed, the method was not considered to be physiologically relevant. Literature reports indicate that the time of residence of food in the small intestine is ~4 hr (±1 hr); consequently, a modified integrated TDF method (AOAC Method 2017.16) was developed. In this method, the incubation time with PAA/AMG is 4 hr, and concentrations of both PAA and AMG were adjusted to ensure that the measured RS values obtained for a number of reference samples were in line with ileostomy data. Under the incubation conditions of AOAC Method 2017.16, the DF values obtained for most RS containing samples were similar to those obtained with AOAC Method 2009.01. One major exception is Fibersym®, for which a DF value of ~60% was obtained as compared to a value of ~30% *w/w* obtained under the extended incubation conditions of AOAC Method 2009.01.

In a recent report on the in vivo digestibility of cross-linked phosphorylated (CLP) starch in ileostomy subjects (Iacovoua et al., 2017), DF output values of 40% *w/w* (average over 10 patients) were obtained for Fibersym® using the Prosky et al. (1985) method. The authors state that "the 40% in vivo RS for CLP wheat starch determined by the Prosky assay to quantitate outgoing fiber in ileostomy subjects is erroneously low" and that the "main cause of the low recovery is due to α-amylase damage to granules of CLP wheat starch in a subject's small intestine." These results and the authors' conclusions are, in fact, consistent with the results obtained with the rapid integrated TDF procedure (AOAC Method 2017.16). Starch granules, including $RS_4$, are digested to different extents by PAA during passage through the human small intestine, highlighting the importance of simulating in vivo digestion conditions in in vitro assays of RS and DF. However, the authors, not satisfied with the experimental results obtained, introduced a "recovery

correction factor" to adjust the experimentally determined fiber values to values in line with what they obtained for native CLP using the Prosky et al. (1985) procedure; a physiologically nonrelevant in vitro assay. The "recovery correction factor" was based on the ratio (~80%) they obtained for starch measured with AOAC Method 996.11 (DMSO format) and the Shukri et al. (2015) method for six 2-hr ileostomy effluent samples apparently chosen at random from their study. It is not clear why the authors simply did not analyze the starch content of the total effluents with the "quantitative" Shukri et al. (2015) method. In contrast to the starch values of ~100% *w*/*w* obtained by Shukri et al. (2015) and Shi et al. (2019) v for Fibersym®, we were able to obtain values no higher than 84% dwb, independent of which assay format was used. In the reported ileostomy study of Iacovoua et al. (2017), the average in vivo recovery of wheat starch was 10.8% *w*/*w*, which is much higher than the <1% *w*/*w* values obtained in other ileostomy studies and in vitro assays. The authors suggest that this high value could be due to glucose that "originated from the hard candy" in the diet. This free glucose should have been separately measured and subtracted from the "total starch" values to obtain an accurate measurement of starch. This candy-derived glucose may also have influenced the reported values for starch in the phosphate cross-linked starch samples. As a concluding remark, the authors state that the Prosky et al. (1985) method must be used for the analysis of some Fibersym®-containing samples, but not for others. As stated previously, we believe that the method is unsuitable for analysis of any of the Fibersym® samples.

## 4.3 | Available carbohydrates

For food labeling in the United States, the only allowed method for labeling of carbohydrates in the nutrition facts panel involves subtracting the amount of crude protein, total fat, moisture, and ash from the total sample weight. Dietary fiber is included in the total carbohydrate value, but can be stated separately on the label. "Net carbohydrates" is determined by subtracting the dietary fiber value from the total carbohydrates value. A major problem in using this method is that of accumulated errors (FAO, 1998). In this paper, a simple method is described for the direct measurement of the available carbohydrates (glucose, fructose, and galactose derived from free glucose, maltodextrins, sucrose, lactose, and digestible starch) by direct measurement of glucose, fructose, and galactose. Such values also allow the estimation of the glycemic index value of a food.

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## SUPPORTING INFORMATION

Additional supporting information may be found online in the Supporting Information section at the end of the article.

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