Megazyme

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RAPID INTEGRATED TOTAL DIETARY FIBER ASSAY PROCEDURE

Including
Resistant Starch
and
Non-Digestible Oligosaccharides

K-RINTDF 01/20

(100 Assays per kit)

AOAC Method 2017.16
ICC Draft Standard 185

GENERAL INTRODUCTION:

The definition for dietary fiber adopted by the Codex Alimentarius Commission (CAC) in June 2009[1] includes carbohydrate polymers that are not hydrolysed by the endogenous enzymes in the small intestine of humans and thus includes resistant starch (RS). This definition also includes oligosaccharides of degrees of polymerisation 3 to 9, but the decision on whether or not to include these oligosaccharides in the dietary fiber value, was left to the discretion of national authorities.

A method designed to support the CAC definition was published in 2007[2], and this method was successfully evaluated in interlaboratory studies[3,4] and approved by AOAC International (2009.01; 2011.25).[3-5] AOAC Method 2009.01 allows the measurement of TDF by summing the quantity of higher molecular weight dietary fiber (HMWDF), which included insoluble dietary fiber (IDF) and soluble dietary fiber that precipitates in the presence of 78% aqueous ethanol (SDFP), with soluble dietary fiber that remains soluble in 78% aqueous ethanol (SDFS). However, application of this method to a range of food products and ingredients over the past 8 years has identified several challenges/concerns: namely,

a) an incubation time with PAA plus AMG of 16 h (the digestion step paralleling the incubation conditions employed in AOAC Method 2002.02 for RS)[4,6] has no physiological basis. A more likely residence time for food in the small intestine is 4 +/- 1 h.[7-9]

b) most commercially available fructo-oligosaccharides (FOS) contain the trisaccharide, fructosyl-β-(2-1)-fructosyl-β-(2-1)-fructose (inulinotriose) which is not measured as dietary fiber (DF) using a Waters Sugar-Pak® HPLC column because it elutes with the disaccharide fraction.

c) under the incubation conditions used in AOAC Method 2009.01, resistant maltodextrins are produced during the hydrolysis of non-resistant starch, and these are incorrectly measured as DF.[8,10,11]

d) the extended incubation time of samples with pancreatic α-amylase/amyloglucosidase (PAA/AMG) results in excessive hydrolysis, and thus under estimation, of phosphate cross-linked starch (RS_4, e.g. Fibersym®)[12] and

e) the use of the preservative, sodium azide, is undesirable on the basis of health concerns to analysts.

In the "Rapid" Integrated TDF procedure (RINTDF) (Figure 1) described here, each of these challenges/limitations of AOAC Methods 2009.01 and 2011.25 has been addressed and resolved.[8] Incubation with higher concentrations of PAA and AMG prevent the formation of resistant maltodextrins (Figure 2), and the shorter incubation time, in line with physiological conditions, yields higher DF values for Fibersym® (RS_4) and Hylon VII® (high amylose maize starch) (Table 1). Replacement of the Waters Sugar-Pak® column with the TOSOH TSKgel® G2500PW_{XL} gel permeation columns[13] has resolved the

problems associated with chromatography of F3 (Figure 3); and the shorter incubation time removes the need to include sodium azide in the incubation buffer. Furthermore, incubation with the higher levels of PAA and AMG used in the RINTDF procedure gives the same SDFS values for all non-digestible oligosaccharides (NDO) except isomaltooligosaccharides, as is obtained with AOAC Method 2009.01 (Table 2). Sample preparation for HPLC has been simplified by removing most salt by deionisation with resins in a tube, followed by complete deionisation using Bio-Rad HPLC deionisation pre-columns.

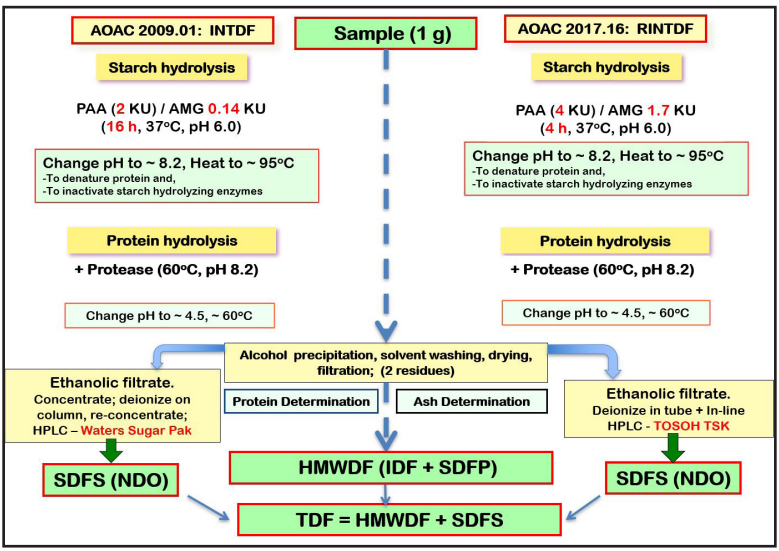

Figure 1. Measurement of TDF using AOAC Methods 2009.01 and 2017.16.

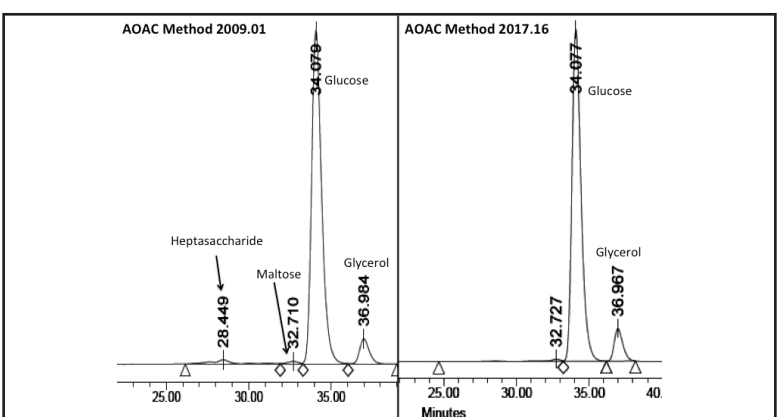

Figure 2. HPLC on TSKgel® G2500PW$_{XL}$ columns of the SDFS fraction obtained from Uncle Ben's Ready Rice incubated according to AOAC Methods 2009.01 and 2017.16. Note the presence of heptasaccharide fraction in the SDFS fraction after running incubations according to AOAC 2009.01 and the absence with AOAC Method 2017.16.

Table 1. Comparison of total dietary fiber values obtained for a range of samples using AOAC Method 2009.01 (Integrated TDF procedure) and AOAC Method 2017.16 (RINTDF assay procedure).

Sample	Total dietary fiber, g/100g	
	AOAC Method 2009.01	AOAC Method 2017.16
Whole meal bread	14.2	13.5
Oat bran	19.5	21.3
Weetabix	12.6	11.6
Kellogg All Bran®	30.5	31.7
Whole wheat pasta	12.7	12.3
Semi-ripe bananas	31.1	31.6
Sweet corn (tinned)	13.1	12.9
Garden peas (tinned)	30.5	31.3
Brocolli	28.5	30.3
Carrots	22.4	23.4
Fibersym®	29.7	60.2
Hylon VII®	49.3	58.8

Table 2. Recovery of oligosaccharides of DP ≥ 3 in original samples and on incubation of the samples according to AOAC 2009.01 and AOAC Method 2017.16 (RINTDF assay procedure).

Sample	Recovery of oligosaccharide of DP > 3 as a percentage of total carbohydrate		
	Original oligosaccharide	AOAC Method 2009.01	AOAC Method 2017.16
Neosugars (FOS)	93.0	92.9	92.8
Raftilose P-95® (FOS)*	91.2	76.2	89.1
Polydextrose	84.3	85.1	82.5
Fibersol 2®	88.5	83.4	82.4
Galactooligosaccharides (GOS)	76.0	70.6	72.0
Xylooligosaccharides (XOS)	78.0	78.6	76.2
Raffinose	99.0	99.0	98.0
AdvantaFiber® (isomaltooligo-saccharides	65.4	29.0	10.8

* a Raftilose P-95 sample with a high content of the trisaccharide, inulotriose.

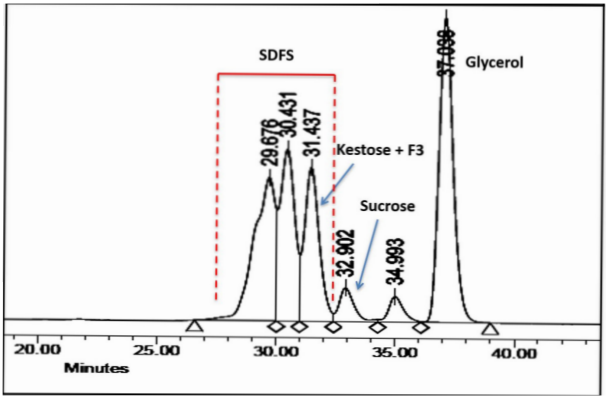

Figure 3. HPLC on TSKgel®G2500PW$_{XL}$ columns of the SDFS fraction obtained on incubation of Raftilose P-95 (200 mg) plus glycerol (100 mg) according to the RINTDF procedure.

A Rapid Integrated Method for the Measurement of Total Dietary Fiber (Including Resistant Starch and Non-Digestible Oligosaccharides (NDO)
(AOAC Method 2017.16)

A. PRINCIPLE:

A rapid integrated procedure (RINTDF) is described for the measurement of total dietary fiber, including RS and SDFS (*i.e.* NDO) of DP ≥ 3 in all foods and food ingredients. This method combines the key attributes of AOAC Official Methods 2002.02, 985.29, 991.43, 2001.03 and 2009.01. Duplicate test portions are incubated with stirring or shaking in the presence of PAA and AMG for 4 h at 37°C in sealed 250 mL bottles (Figures 4 & 5, page 18). During this step, non-resistant starch is solubilised and hydrolysed to D-glucose and traces of maltose. The reaction is terminated by adjustment of the pH to 8.2 (at which pH AMG has no action) and then heating to ~ 95°C to inactivate both AMG and PAA. Protein in the sample is denatured and digested with protease. Specific dietary fiber fractions are measured as follows:

i. Total High Molecular Weight Dietary Fiber (HMWDF) and SDFS determination.

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

ii. Insoluble Dietary Fiber (IDF), SDFP and SDFS determination.

IDF is recovered by filtration of the aqueous reaction mixture and the residue is washed, dried and weighed. SDFP in the filtrate is precipitated with ethanol or industrial methylated spirits (IMS), recovered, dried and weighed. Both the IDF and SDFP residues are corrected for protein, ash and blank values for the final calculation of the IDF and SDFP values. The aqueous ethanol filtrate recovered on precipitation and removal of SDFP, namely SDFS, is concentrated, deionised and analysed by HPLC.

The enzymes used in these methods are of very high purity; they are effectively devoid of contaminating enzymes active on β-glucan, pectin and arabinoxylan (Table 3). NDO such as FOS and GOS are not hydrolysed (Table 2), and the degree of hydrolysis of Polydextrose® is in line with the information provided by the supplier.

Table 3. Control enzymes are analysed to ensure the presence of appropriate enzyme activity and absence of undesirable enzyme activity (these controls are available in the kit **K-TDFC** from Megazyme). Controls are analysed through the entire procedure.

Test sample	Activity tested	Sample weight (g)	Expected recovery (%)
Citrus pectin	Pectinase[c]	0.1	85[c]
β-Glucan (barley)	β-Glucanase[a]	0.1	97
Wheat starch	α-Amylase[b]	1.0	0-1
Casein	Protease[b]	0.3	0-1
High amylose starch[d] (Hylon VII)	α-Amylase	1.0	~ 59
Galactan (larch)	Pectinase[c]	0.1	~ 86[c]

[a] This activity should not be present in the tests.
[b] This activity should be fully functional in the tests.
[c] Low values are mainly due to the moisture content of samples. Similar values are obtained with no enzymes in the incubations.
[d] This material contains a high level of enzyme resistant starch. This DF value is higher than that obtained with AOAC Method 991.43 (29.3%).

KITS:

Bottle 1: (x2) Mixture of purified PAA (40 KU/g) and AMG (17 KU/g) pancreatic α-amylase; 5.2 g.
Stable for > 5 years stored dry below -10°C.

NOTE: For some individuals, these powdered enzymes are highly allergenic. Thus, they should be weighed and handled **ONLY** in a fume cupboard; see *[C(d)]*.

Bottle 2: Purified protease (E-BSPAMS) (10.5 mL, 350 tyrosine U/mL in 3.2 M ammonium sulphate).
Stable for > 3 years at 4°C.

Bottle 3: LC Retention Time Standard [maltodextrins plus maltose (4:1 ratio)], approx. 5 g.
Stable for > 3 years; store sealed at room temperature.

Bottle 4: (x2) Glycerol standard solution (100 mg/mL, 55 mL).
Stable for > 4 years; store sealed at 4°C.

Bottle 5: D-Glucose/glycerol (Glu/Gly) standard solution (10 mg/mL of each in 0.02% w/v sodium azide).
Stable for > 4 years; store sealed at 4°C.

B. APPARATUS REQUIRED:

a. ***Grinding mill.***— Centrifugal, with 12-tooth rotor and 0.5 mm sieve, or similar device. Alternatively, a cyclone mill can be used for small test laboratory samples provided the mill has sufficient air flow or other cooling to avoid overheating of samples.

b. ***Digestion Bottles.***— 250 mL Fisherbrand® soda glass, wide mouth bottles with with polyvinyl lined cap (cat. no. FB73219) (www.fisher.co.uk/supplies/fisherbrand.html).

c. ***Fritted crucible.***— Gooch, fritted disk, Pyrex® 50 mL, pore size coarse, ASTM 40-60 mm, Corning® No. 32940-50C.

Prepare four replicates for each sample as follows:

i. Ash overnight at 525°C in muffle furnace, cool furnace to 130°C before removing crucibles to minimise breakage.
ii. Remove any residual Celite® and ash material by using a vacuum.
iii. Soak in 2% cleaning solution, *[C(o)]* at room temperature for 1 h.
iv. Rinse crucibles with water and deionised water.
v. For final rinse, use 15 mL acetone and air dry.
vi. Add approx. 1.0 g Celite® to dried crucibles and dry at 130°C to constant weight.
vii. Cool crucible in desiccator for approx. 1 h and record mass of crucible containing Celite®.

d. ***Filtering flask.***— heavy-walled, 1 L Büchner flask with side arm (Figure 6, page 19).

e. ***Rubber ring adaptors.***— for use to join crucibles with filtering flasks (Figure 6, page 19).

f. ***Vacuum source.***— vacuum pump or aspirator with regulator capable of regulating vacuum (e.g. Edwards XDS 10; single-phase 115/230V; product code: A726-01-903).

g. ***Water bath(s).***— 2mag Mixdrive 15® submersible magnetic stirrer with a 30 x 7 mm stirrer bar, set at 170 rpm in a bath heated with an immersion heater (e.g. Lauda Alpha®) (Figure 4, page 18). Alternatively, rotary motion (150 rpm), large-capacity (20-24 L) with covers; capable of maintaining temperature of 37 ± 1°C and 60 ± 1°C; (e.g. Grant® OLS 200 shaking incubation bath) (Figure 5, page 18).

h. ***Balance.***— 0.1 mg readability, accuracy and precision.

i. ***Ovens.***— two, mechanical convection, set at 105 ± 2°C and 130 ± 3°C.

j. ***Timer.***

k. ***Desiccator.***— airtight, with silica gel or equivalent desiccant. Desiccant dried biweekly overnight in 130°C oven.

l. ***pH meter.***

m. ***Positive displacement pipettor.***— e.g. Eppendorf Multipette®

- with 25 mL Combitip® (to dispense 5 mL aliquots of PAA/AMG preparation, 3 mL aliquots of 0.75 M Tris base solution and 4 mL aliquot of 2 M acetic acid).
- with 5.0 mL Combitip® (to dispense 0.1 mL of protease solution).

n. ***Dispensers.***— to dispense 15 ± 0.5 mL of 78% v/v EtOH (or IMS), 95% v/v EtOH and acetone, and 35 ± 0.2 mL of buffer.

o. ***Cylinder.***— Graduated, 100 mL and 500 mL.

p. ***Magnetic stirrers and stirring bars.***— 7 x 30 mm; plain magnetic stirrer bars; VWR International; cat no. 442-0269.

q. ***Rubber policeman spatulas.***— VWR International; cat. no. 53801-008) (Figure 6, page 19).

r. ***Muffle furnace.***— 525 ± 5°C.

s. ***Polypropylene tubes.***— Sarstedt polypropylene tube; 13 mL, 101 x 16.5 mm (cat no. 60.541.685 https://www.sarstedt.com/

en/search/?id=77&L=1&q=60.641.685&x=2&y=3 (accessed 13th December, 2019).

t. ***Liquid Chromatograph (LC).***— With oven to maintain a column temperature of 80°C and a 50 μL injection loop. System must separate maltose from maltotriose.

u. ***Guard column (or pre-column).***— TSK® guard column PW_{XL} 6.0 mm id x 4 cm (Tosoh Corp., Cat. no. TSKgel® $G2500PW_{XL}$, https://www.separations.us.tosohbioscience.com/HPLC_Columns/id-8322/TSKgel_G2500PWxl).

v. ***Cation and anion exchange guard columns (deionising columns).***— Cation and anion exchange guard columns, H^+ and CO_2^{3-} forms respectively (Bio-Rad Laboratories, Cat. No. 125-0118, includes one cation and one anion cartridge), with guard column holder (Bio-Rad Laboratories, Cat. No. 125-039) to hold the two guard column cartridges in series cation cartridge preceeding anion cartridge)[14] (Figure 7, page 19).

w. ***LC column.***— Two LC columns connected in series. TSKgel® $G2500PW_{XL}$, 7.8 mm id x 30 (Tosoh Corp; https://www.separations.us.tosohbioscience.com/HPLC_Columns/id-8322/TSKgel_G2500PWxl); flow rate 0.5 mL/min; column temp. 80°C; run time 60 min to assure the column is cleaned out (Figure 8, page 19).

x. ***Detector.***— Refractive index (RI); maintained at 50°C.

y. ***Data integrator or computer.***— For peak area measurement.

z. ***Filters for disposable syringe.***— Millipore Millex® Syringe Driven Filter Unit 0.45 μm (low protein binding Durapore PVDF), 25 mm or 13 mm or equivalent.

aa. ***Filters for water.***— Millipore, 0.45 μm Durapore® Membrane Filters type HVLP, 47 mm.

bb. ***Filter apparatus.***— To hold 47 mm, 0.45 μm filter, *[B(aa)]*; to filter larger volumes of water.

cc. ***Syringes.***—10 mL, disposable, plastic.

dd. ***Syringes.***— Hamilton® 100 μL, 710SNR syringe.

ee. ***Rotary evaporator.***— Heidolph Laborota® 4000 or equivalent.

ff. ***Microfuge centrifuge.***— Capable of 13,000 rpm.

gg. ***Thermometer.***— Capable of measuring to 110°C.

C. REAGENTS:

a. ***Ethanol (or IMS) 95% v/v.***

b. ***Ethanol (or IMS) 78% v/v.***— Place 180 mL deionised water into a 1 L volumetric flask. Dilute to volume with 95% v/v ethanol (or IMS). Mix.

c. ***Acetone, reagent grade.***

d. ***Stock PAA / AMG solution.***— PAA (4 KU/5 mL) plus AMG (1.7 KU/5 mL). Immediately before use, add 1.0 g of PAA/AMG powder mixture (**Bottle 1**, page 6) to 50 mL of sodium maleate buffer *[C(g)]* and stir on a magnetic stirrer for 5 min. Store on ice during use. Use within 4 h of preparation.

NOTE 1: If an analyst is allergic to powdered PAA and/or AMG, engage an analyst who is not allergic to prepare the powdered enzymes as an ammonium sulphate suspension as follows: gradually add 5 g of PAA/AMG powder mix (PAA 40 KU/g plus AMG 17 KU/g; **Bottle 1**, page 6) to 70 mL of cold, distilled water in a 200 mL beaker on a magnetic stirrer in a fume cupboard and stir until the enzymes are completely dissolved (approx. 5 min). Add 35 g of granular ammonium sulphate and dissolve by stirring. Adjust the volume to 100 mL with ammonium sulphate solution (50 g/100 mL). Stable at 4°C for 3 months.

e. ***Protease (50 mg/mL; 350 Tyrosine Units/mL) in 3.2 M ammonium sulphate solution.***— Use the contents of **Bottle 2** (page 6) as supplied. Swirl the contents gently before use to give uniform suspension. Protease must be devoid of α-amylase and essentially devoid of β-glucanase and β-xylanase. Store on ice during use. Stable for > 3 years at 4°C.

f. ***Deionised water.***

g. ***Sodium maleate buffer.***— 50 mM, pH 6.0 plus 2 mM $CaCl_2$. Dissolve 11.6 g of maleic acid in 1600 mL of deionised water and adjust the pH to 6.0 with 4 M (160 g/L) NaOH solution. Add 0.6 g of calcium chloride dihydrate ($CaCl_2.2H_2O$), dissolve and adjust the volume to 2 L. Store in a well-sealed Duran® bottle and add two drops of toluene to prevent microbial infection. Stable for ~ 1 year at 4°C.

h. ***Tris solution, 0.75 M.***— Add 90.8 g of Tris buffer salt (Megazyme cat. no. **B-TRIS500**) to approx. 800 mL of deionised water and dissolve. Adjust the pH to 11 and the volume to 1 L. Stable for ~ 1 year at room temperature.

i. ***Acetic acid solution, 2 M.***— Add 115 mL of glacial acetic acid

(Sigma W200611-1KG-K) to a 1 L volumetric flask. Dilute to 1 L with deionised water. Stable for > 1 year at room temperature.

j. ***Sodium azide solution (0.02% w/v).***— Add 0.2 g of sodium azide to 1 L of deionised water and dissolve by stirring.

NOTE 2: do not add sodium azide to solutions of low pH. Acidification of sodium azide releases a poisonous gas. Handle sodium azide with caution only after reviewing SDS, using appropriate personal protective gear and laboratory hood). Stable for > 4 years at room temperature.

k. ***D-Glucose / Glycerol LC standard for determination of HPLC Rf value.***— 10 mg/mL of each in 0.02% w/v sodium azide. Provided as **Bottle 5** (page 6). Use as supplied. Stable for > 4 years at 4°C.

l. ***Glycerol (Internal standard for TSK® gel permeation column).***— 100 mg/mL containing sodium azide (0.02% w/v). Use the contents of **Bottle 4** (page 6) as supplied. Stable for > 4 years at 4°C.

m. ***LC retention time standard.***— Standard having the distribution of oligosaccharides (DP > 3) corn syrup solids (DE 25; Matsutani Chemical Industry Co., Ltd., Itami City, Hyogo, Japan; (www.matsutani.com) plus maltose in a ratio of 4:1 (w/w). Dissolve 2.5 g of mixture (**Bottle 3**, page 6) in 80 mL of 0.02% sodium azide solution and transfer to 100 mL volumetric flask. Pipette 10 mL of internal standard *[C(l)]* into the flask. Bring to volume with 0.02% sodium azide solution *[C(j)]*. Transfer solutions to 50 mL polypropylene storage bottles. Stable for > 1 year at room temp; stable for > 4 year below -10°C.

n. ***pH standards.***— Buffer solutions at pH 4.0, 7.0 and 10.0.

o. ***Cleaning solution.***— Micro-90® (International Products Corp., USA, cat. no. M-9033, www.ipcol.com/shopexd.asp?id=15). Make a 2% solution with deionised water.

p. ***Cation exchange resin.***— Amberlite® FPA53 (OH^-) resin (Megazyme cat. no. **G-AMBOH**), ion exchange capacity 1.6 meq/mL (min) or equivalent (R-OH^- exchange capacity data supplied by manufacturer).

q. ***Anion exchange resin.***— Ambersep® 200 (H^+) resin or equivalent, (Megazyme cat. no. **G-AMBH**), ion exchange capacity: 1.6 meq/mL (minimum) or equivalent (R-H^+ exchange capacity data supplied by manufacturer).

r. ***Celite®.***— acid-washed, pre-ashed (Megazyme cat. no. **G-CELITE**).

D. PREPARATION OF TEST SAMPLES:

Collect and prepare samples as intended to be eaten, *i.e.* baking mixes should be prepared and baked, pasta should be cooked *etc.* Defat per AOAC 985.29 if > 10% fat. For high moisture samples (> 25%) it may be desirable to freeze dry. Grind ~ 50 g in a grinding mill *[B(a)]* to pass a 0.5 mm sieve. Transfer all material to a wide mouthed plastic jar, seal, and mix well by shaking and inversion. Store in the presence of a desiccant.

E. ENZYME PURITY:

To ensure absence of undesirable enzymatic activities and effectiveness of desirable enzymatic activities, run standards (Megazyme cat. no. **K-TDFC**) after the enzyme has been stored for more than 12 months.

F. ENZYME DIGESTION OF SAMPLES:

(1) Blanks

With each set of assays, run two blanks along with samples to measure any contribution from reagents to residue.

(2) Samples

(a) ***Accurately weigh*** approx. 1 g sample, correct to the third decimal place, in duplicate into 250 mL Fisherbrand glass® bottles *[B(b)]*. Record the weight.

(b) ***Addition of Enzymes.***— Wet the sample with 1.0 mL of 95% EtOH (or IMS) *[C(a)]* and add 35 mL of maleate buffer *[C(g)]* to each bottle. Cap the bottles. Transfer the bottles to a Grant OLS 200 shaking incubation bath (or similar) *[B(g)]* and secure the bottles in place with springs or polypropylene support in the shaker frame (Figure 5, page 18). Allow the solution to equilibrate to temperature for 5 min. Alternatively, use a 2mag Mixdrive 15® submersible magnetic stirrer *[B(g)]* with a 7 x 30 mm stirrer bar *[B(p)]* added to each bottle (Figure 4, page 18).

(c) ***Incubation with PAA/AMG solution.***— Add 5 mL of PAA/AMG solution *[C(d)]*, cap the bottles and incubate the reaction solutions at 37°C and 150 rpm in orbital motion in a shaking water bath *[B(g)]*; or at 170 rpm on a 2mag Mixdrive 15® submersible magnetic stirrer (to ensure complete suspension) for ***exactly*** 4 h. **NOTE:** If using the $(NH_4)_2SO_4$ suspension of this enzyme preparation *[C(d)]*, add 2 mL of enzyme suspension and 3 mL of sodium maleate buffer *[C(g)]*.

(d) ***Adjustment of pH to approx. 8.2 (pH 7.9-8.4), Inactivation of α-amylase and AMG.***— After 4 h, remove all sample bottles

from the shaking water bath and immediately add 3.0 mL of 0.75 M Tris buffer solution *[C(h)]* to terminate the reaction (At the same time, if only one incubation bath is available, increase the temperature of the bath to 60°C in readiness for the protease incubation step). Slightly loosen the caps of the sample bottles and immediately place the bottles in a water bath (non-shaking) at 95-100°C, and incubate for 20 min with occasional shaking (by hand). Using a thermometer, ensure that the final temperature of the bottle contents is > 90°C (checking of just one bottle is adequate).

(e) ***Cool.***— Remove all sample bottles from the boiling water bath (use appropriate gloves) and place in the water bath set at 60°C and allow the temperature to equilibrate to approx. 60°C over 10 min.

(f) ***Protease treatment.***— Add 0.1 mL of protease suspension (**Bottle 2**, page 6) *[C(e)]* with a positive displacement dispenser (solution is quite thick). Incubate at 60°C for 30 min.

(g) ***pH adjustment.***— Adjust pH by adding 4.0 mL of 2 M acetic acid *[C(i)]* to each bottle and mix. This gives a final pH of approx 4.3.

(h) ***Internal standard.***— Add 1.0 mL of glycerol internal standard solution (100 mg/mL; **Bottle 4**, page 6); *[C(l)]* to each incubation bottle and mix well.

(i) ***Proceed to step [G(a)]*** for determination of **HMWDF (IDF + SDFP)** or to ***step [H(a)]*** for determination of **IDF, SDFP & SDFS.**

NOTE:
If available carbohydrates are to be determined, accurately transfer 0.5 mL of incubation solution to a Microfuge tube and centrifuge at 13,000 rpm for 3 min. Transfer 0.2 mL to a 13 mL (101 x 16.5 mm) polypropylene tube and add 5 mL of distilled water, cap the tube and store below -10°C awaiting analysis of available carbohydrate using the Megazyme Available Carbohydrate Assay Kit (**K-AVCHO**).

G. DETERMINATION of HMWDF (IDF plus SDFP): (same procedure as for AOAC Method 2009.01):

(a) ***Precipitation SDFP.***— Preheat the sample to 60°C and add 220 mL of 95% (v/v) EtOH or IMS *[C(a)]* measured at room temperature and pre-heated to 60°C. Mix thoroughly and allow the precipitate to form at room temperature for 60 min (overnight precipitation is acceptable).

(b) ***Filtration setup.***— Tare crucible containing Celite® *[B(c)]* to the nearest 0.1 mg. Wet and redistribute the bed of Celite® in the crucible, using 15 mL of 78% (v/v) EtOH or IMS *[C(b)]* from wash bottle. Apply suction to crucible to draw Celite® onto fritted glass as an even mat (Figure 6, page 19). Discard the filtrate.

(c) ***Filtration.***— Using vacuum, filter precipitated enzyme digest ***[G(a)]*** through the crucible. Using a wash bottle with 78% (v/v) EtOH or IMS quantitatively transfer all remaining particles to crucible and wash the residue successively with two 15 mL portions of 78% (v/v) EtOH (or IMS) *[C(b)]*. Retain the filtrate and washings and adjust the volume to 300 mL with 78% EtOH and proceed to ***step [I(a)]*** on page 15 for determination of **SDFS**.

(d) ***Wash.***— Using a vacuum, wash residue sequentially with two 15 mL portions of the following: 78% (v/v) EtOH (or IMS) *[C(b)]*, 95% (v/v) EtOH (or IMS) *[C(a)]* and acetone *[C(c)]*. Discard these washings. Draw air through the crucible for at least 2 min to ensure all acetone is removed before drying the crucibles in an oven.

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

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

(g) ***Protein and ash determination.***— The residue from one crucible is analysed for protein and the second residue of the duplicate is analysed for ash. Perform protein analysis on residue using Kjeldahl or combustion methods (**Caution** should be exercised when using a combustion analyser for protein in the residue. Celite® volatilised from the sample can clog the transfer lines of the unit). Use 6.25 factor for all cases to calculate mg of protein. For ash analysis, incinerate the second residue for 5 h at 525°C *[B(r)]*. Cool in desiccator and weigh to nearest 0.1 mg. Subtract crucible and Celite® weight to determine ash.

(h) ***Calculation of HMWDF (IDF + SDFP) and SDFS.***— Proceed to ***step [J]*** (page 17).

H. DETERMINATION of IDF and SDFP separately: *(same procedure as for AOAC Method 2011.25):*

IDF

(a) ***Filtration setup.***— Tare crucible containing Celite® *[B(c)]* to nearest 0.1 mg. Wet and redistribute the bed of Celite® in the crucible, using 15 mL of 78% (v/v) EtOH (or IMS) *[C(b)]* from wash bottle. Apply suction to crucible to draw Celite® onto the fritted glass as an even mat (Figure 6, page 19). Discard the filtrate.

(b) ***Filtration.***— Using vacuum, filter the enzyme digest from ***step [F(2)(a)]*** through the crucible. Using a wash bottle with 60°C deionised water rinse the incubation bottle with a minimum volume of water (approx. 10 mL) and use a rubber policeman (spatula) *[B(q)]* to dislodge all particles from the walls of the container. Transfer this suspension to the crucible. Wash the incubation bottle with a further 10 mL of water at 60°C and again transfer to the crucible. Collect the combined filtrate and washings and adjust the volume to 70 mL and retain this for determination of **SDFP *[H(f)]*** and **SDFS *[H(g)]***.

(c) ***Wash.***— Using a vacuum, wash the residue successively with two 15 mL portions of the following: 78% (v/v) EtOH (or IMS) *[C(b)]*, 95% (v/v) EtOH (or IMS) *[C(a)]* and acetone *[C(c)]*. Discard the washings. Draw air through the crucible for at least 2 min to ensure all acetone is removed (to prevent explosion hazard) before drying crucibles in oven.

(d) ***Dry crucibles*** containing residue overnight in 105°C oven.

(e) ***Cool crucibles and determination of IDF***. Cool crucibles and determine residue mass as described in *[G(e)]* to *[G(f)]*. Determine protein and ash as described in *[G(g)]* and subtract from residue weight. Calculate IDF as described in **step *[J]*** (see **NOTE**, page 17).

SDFP

(f) ***Precipitation of SDFP.***— Pre-heat the filtrate of each sample from *[H(b)]* (approx. 70 mL) to 60°C and add 320 mL of 95% (v/v) EtOH or IMS *[C(a)]* (measured at room temperature and then pre-heated to 60°C) and mix thoroughly. Allow the precipitate to form at room temperature for 60 min (overnight precipitation is acceptable).

(g) ***Filtration and recovery of SDFP and SDFS.***— Filter the suspension and recover the residue, analyse this for protein and ash and calculate **SDFP** as described in **steps *[G(b)]*** to ***[G(g)]***. Retain the filtrate and washings (approx. 420 mL) and proceed to **step *[I(a)]*** for determination of SDFS.

I. DETERMINATION OF SDFS:

Note: Proper deionisation is an essential part of obtaining quality chromatographic data. Refer to Figure 9 (page 20) to see patterns of glycerol and D-glucose in the presence and absence of buffer salts. To ensure that the resins being used are of adequate deionising capacity, add 0.1 mL of protease suspension (**Bottle 2**, page 6) to 40 mL of maleate buffer *[C(g)]* along with 3.0 mL of 0.75 M Tris base solution *[C(h)]*, 4.0 mL of 2 M acetic acid *[C(i)]*, 1 mL of glycerol internal standard (100 mg/mL; **Bottle 4**, page 6) *[C(l)]* and 1 mL of D-glucose solution (100 mg/mL). Concentrate this solution to dryness on a rotary evaporator and re-dissolve the residue in 32 mL of deionised water. To 5 mL of this solution in a 13 mL polypropylene tube *[B(s)]*, add 1.5 g of Amberlite® FPA53 (OH^-) resin and 1.5 g of Ambersep® 200 (H^+) (Figure 8, page 19), cap the tube and invert the contents regularly over 5 min. Allow the resin to settle and remove the supernatant (1.5-2.0 mL) with a syringe *[B(cc)]* and filter through a polyvinylidene fluoride filter, pore size 0.45 μm *[B(z)]*. Inject an aliquot (50 μL) of this solution onto the TSK columns (with deionising pre-column in place). A pattern similar to that shown in Figure 9c (page 20) should be obtained, *i.e.* no salt peaks should be evident.

(a) ***Filtrate recovery and concentration.***— {Set aside the filtrate from one of the sample duplicates *[G(c)]* or *[H(g)]* to use if duplicate SDFS data is desired}. Transfer one quarter of the filtrate *[G(c)]* or *[H(g)]* of the second sample duplicate, {*i.e.* ~ 75 mL of [G(c)] or ~ 105 mL of [H(g)]} to a 500 mL evaporator flask and evaporate to dryness under vacuum at 60°C. Redissolve in 8 mL of deionised water.

(b) ***Deionisation of sample.***— Transfer 5 mL of sample concentrate from *step [I(a)]* to a 13 mL polypropylene tube *[B(s)]* (quantitative transfer is not required as the sample contains glycerol internal standard). Add ~ 1.5 g of Amberlite® FPA53 (OH^-) resin *[C(p)]* and ~ 1.5 g of Ambersep® 200 (H^+) *[C(q)]* resin to the tube and invert the tube contents over 3-4 min (Figure 8, page 19).

(c) ***Preparation of samples for LC analyses.***— Transfer the solution to a 10 mL disposable syringe *[B(cc)]* and filter through a 0.45 mm filter *[B(z)]*. Alternatively, transfer 1 mL of the solution to a microfuge centrifuge tube and centrifuge *[B(ff)]* at 13,000 rpm for 3 min. Use a 100 μL LC glass syringe *[B(dd)]* to fill the 50 μL injection loop on the LC *[B(t)]*. A single analysis of the sample is adequate. Columns: Two TOSOH TSK gel permeation columns *[B(w)]* with deionising pre-column *[B(v)]*. Solvent: microfiltered *[B(bb)]*, distilled water. Flow rate: 0.5 mL/min; 60 min per run.

Temperature: 80°C, Figure 7, page 19).

(d) ***Determine the response factor for D-glucose.***— Because D-glucose provides an LC refractive index (RI) response equivalent to the response factor for the non-digestible oligosaccharides that make up SDFS, D-glucose is used to calibrated the LC and the response factor is used for determining the mass of SDFS. Use a 100 μL LC syringe to fill a 50 μL injection loop with the D-glucose/glycerol internal standard solution (**Bottle 5**, page 6). Inject in duplicate. Calculate the response factor according to ***[J(b)(1)]***.

(e) ***Calibrate the area of chromatogram to be measured for SDFS.***— Use a 100 μL LC syringe *[B(dd)]*, to fill the 50 μL injection loop with retention time standard **Bottle 3** (page 6) *[C(m)]*. Inject in duplicate. Determine demarcation point between DP 2 and DP 3 oligosaccharides (disaccharide maltose versus higher oligosaccharides) (Figure 3, page 4).

(f) ***Determine peak area of SDFS*** **(PA_{SDFS})** ***and internal standard*** **(PA_{IS})** ***in chromatograms of sample extracts.***— Inject sample extracts *[I(c)]* on LC. Record area of all peaks of DP greater than the DP2/DP3 demarcation point as PA_{SDFS}. Record the peak area of internal standard as PA_{IS}.

(g) ***Proceed to step [J(b)(2)]***.

J. CALCULATIONS of Total Dietary Fiber as HMWDF (IDF + SDFP) + SDFS:

(a) HMWDF (IDF + SDFP) (by gravimetry).

(1) Blank (B, mg) determination.

$$B\ (mg) = \frac{BR_1 + BR_2}{2} - P_B - P_A$$

where BR_1 and BR_2 = residue mass (mg) for duplicate blank determinations respectively; P_B and P_A = mass in mg of protein and ash respectively, determined on first and second blank residues'

(2) [IDF + SDFP] determination.

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

$$[IDF + SDFP]\ g/100\ g = \frac{[IDF+SDFP]\ mg/100\ g}{1{,}000}$$

where R_1 = residue mass 1 from M_1 in mg; R_2 = residue mass 2 from M_2 in mg; M_1 = test portion mass 1 in g; M_2 = test portion mass 2 in g; P_A = ash mass from R_1 in mg; P_B = protein mass from R_2 in mg; B = determined value for the Blank ***[J(a)(1)]*** in mg.

NOTE: The same calculations are performed when IDF and SDFP are determined separately [see **H(a)** and **H(f)**].

(b) SDFS (by HPLC).

(1) Determination of D-glucose response factor.

Obtain the values for the peak areas of D-glucose and internal standard (glycerol) from duplicate chromatograms. The ratio of peak area of D-glucose/peak area of glycerol to the ratio of the mass of D-glucose/mass of glycerol is the "response factor." The average response factor for D-glucose is approximately 0.82 verses glycerol.

$$\text{Response factor (Rf)} = \frac{PA_{IS}}{PA_{Glu}} \times \frac{Wt_{Glu}}{Wt_{IS}}$$

where PA_{Glu} = peak **area** D-glucose; PA_{IS} = peak **area** internal standard (glycerol); Wt_{Glu} = mass of D-glucose in 1 mL of D-glucose/glycerol standard (10 mg); Wt_{IS} = mass of internal standard (glycerol) in 1 mL of D-glucose/glycerol standard (10 mg).

(2) Determination of SDFS.

$$\text{SDFS (mg/100g)} = \frac{Rf \times Wt_{IS} \times PA_{SDFS}}{PA_{IS}} \times \frac{100}{M}$$

$$\text{SDFS (g/100g)} = \frac{\text{SDFS (mg/100g)}}{1{,}000}$$

where Rf = the response factor; Wt_{IS} = mg of internal standard contained in 1 mL of glycerol internal standard solution (100 mg/mL, *i.e.* 100 mg) pipetted into sample before filtration; PA_{SDFS} = the peak area of the SDFS fraction; PA_{IS} = the peak area of the glycerol internal standard; M = the test portion mass (M_1 or M_2) in grams of the sample whose filtrate concentrated and analyzed by LC.

(c) Total Dietary Fiber.

Total dietary fiber (g/100g) = [IDF + SDFP] g/100g) + SDFS (g/100g)

NOTE: These calculations can be simplified by using the Megazyme ***Mega-Calc***™, downloadable from where the product appears in the Megazyme web site (www.megazyme.com).

Figure 4.
2mag Mixdrive 15® submersible magnetic stirrer in a custom made water bath. This allows stirring of 15 samples at controlled speed (170 rpm) and 37°C.

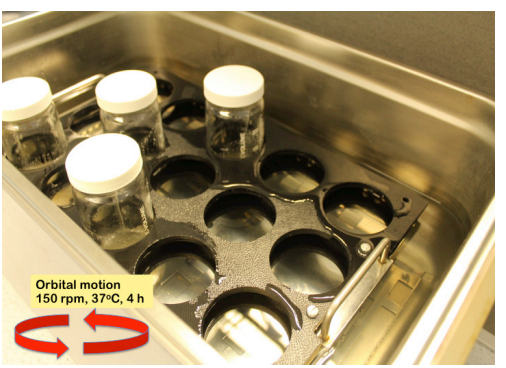

Figure 5.
Grant OLS 200® shaking incubation bath set at 37°C showing custom made polypropylene holder for Fisherbrand® 250 mL bottles.

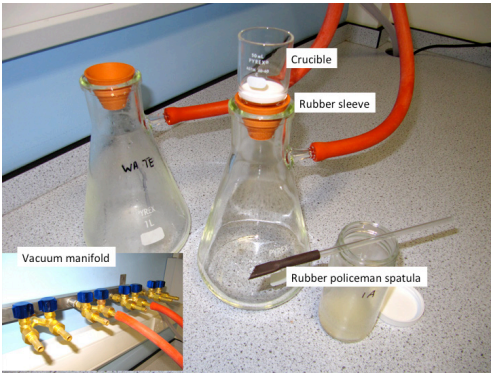

Figure 6.
Buchner flask, crucible, rubber sleeve and vacuum manifold arrangement for filtration of dietary fiber samples. Picture also shows sample bottle and rubber policeman on a glass rod.

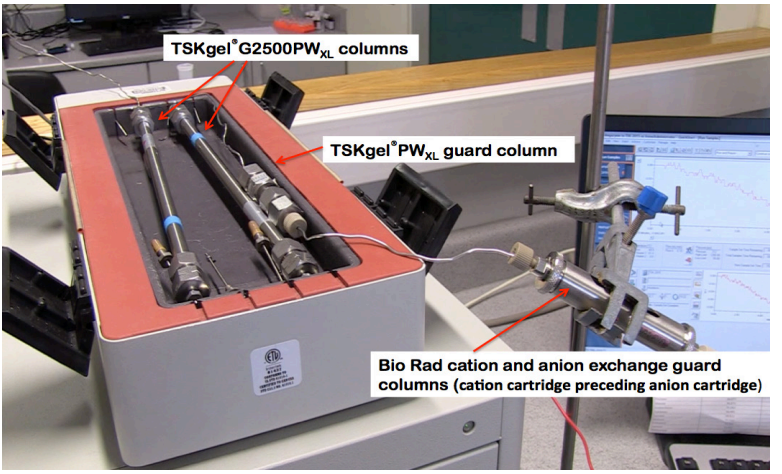

Figure 7. In-line deionisation of samples for chromatography on TSKgel® G2500PW$_{XL}$ HPLC columns.[14]

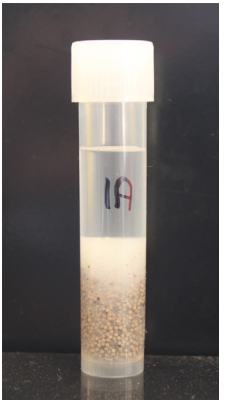

Figure 8.
Deionisation of samples for HPLC. 5 mL of concentrated eluate mixed with 1.5 g of Amberlite® FPA53 (OH^-) and 1.5 g of Ambersep® 200 (H^+) resins.

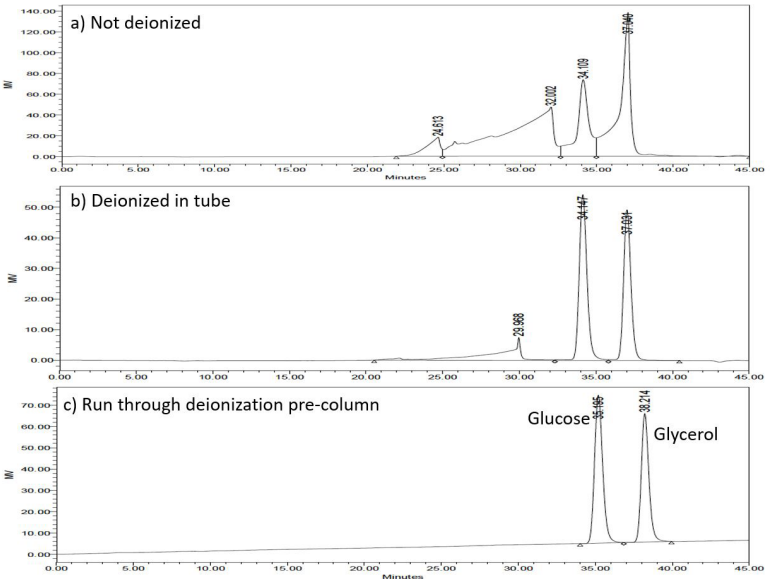

Figure 9.
Chromatography on TSKgel®G2500PW$_{XL}$ HPLC columns of

a) a non-deionised mixture of glucose and glycerol in RINTDF incubation buffers;

b) the same sample after deionising 5 mL with 1.5 g of Amberlite® FPA53 (OH^-) and 1.5 g of Ambersep® 200 (H^+) resins (as shown in Figure 8); and

c) sample "b" on the same TSK columns, but also with deionising pre-columns in place.[14]

INTERLABORATORY EVALUATION of the RINTDF ASSAY PROCEDURE:

The RINTDF method described in this data booklet is an update of AOAC Method 2009.01 and addresses each of the issues identified by analysts in using that method over the past 8 years. This method has been subjected to interlaboratory evaluation under the auspices of AOAC International and the International Association of Cereal Science and Technology (ICC).[15]

Thirteen laboratories participated in the study, with all laboratories returning valid assay data for most of the 16 test portions (8 blind duplicates) consisting of samples with a range of content of traditional dietary fibers, resistant starch and non-digestible oligosaccharides. The dietary fiber content of the 8 test pairs ranged from 6.90 to 60.37 g/100 g. TDF was calculated as the sum of insoluble dietary fiber (IDF) plus SDFP, measured gravimetrically and SDFS measured by HPLC. The results from two laboratories were excluded from the statistical evaluation for reasons described in reference 15). For the remaining 11 laboratories, the repeatability standard deviation (s_r) ranged from 0.27 to 0.76 g/100 g and the reproducibility standard deviation (s_R) ranged from 0.54 to 3.99 g/100 g. The repeatability relative standard deviation (RSD_r) ranged from 1.22 to 6.52% and the reproducibility relative standard deviation (RSD_R) ranged from 2.14 to 10.62% (Table 4). These statistical values are consistent with those reported for analysis of similar samples with other dietary fiber assay formats.[3] On the basis of the results obtained, the RINTDF method was accepted by AOAC International as Method 2017.16 and by ICC as Draft Standard 185.

Table 4. Interlaboratory study results for total dietary fiber in foods (AOAC Method 2017.16). Statistical evaluation according to AOAC International statistics format.

Sample/ parameter	A&D	B&F	C&J	E&H	G&N	I&M	K&O	L&P
No. labs/analysts	10	11	11	10	11	11	11	11
Mean, (g/100 g)	60.37	23.80	29.22	6.90	16.15	19.43	21.02	10.82
S_r (g/100 g)	0.76	0.60	0.36	0.27	0.42	0.30	0.41	0.71
S_R (g/100 g)	3.99	0.72	0.63	0.73	0.79	1.15	0.54	0.91
$\%RSD_r$	1.25	2.52	1.22	3.88	2.59	1.54	1.93	6.52
$\%RSD_R$	6.62	3.03	2.14	10.62	4.88	5.94	2.57	8.46

Samples: A&D = Phosphate cross-linked starch (Fibersym®); B&F = kidney beans (canned, washed and lyophilized); C&J = bran cereal; E&H = Defatted cookies containing FOS; G&N = oat bran; I&M = defatted cookies containing polydextrose and RS_2; K&O = dark rye crispbread; L&P = whole meal bread.

S_r: repeatability; RSD_r: repeatability relative standard deviation; S_R: reproducibility; and RSD_R: reproducibility relative standard deviation

K. REFERENCES:

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