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INTEGRATED TOTAL DIETARY FIBER

ASSAY PROCEDURE

INCLUDING
RESISTANT STARCH
AND NON-DIGESTIBLE
OLIGOSACCHARIDES

K-INTDF 04/20

AOAC Method 2009.01 & 2011.25

&

AACC Method 32-45.01 & 32-50.01

(100 Assays per Kit)

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GENERAL INTRODUCTION:

It is generally believed[1-3] that an increased consumption of dietary fiber (DF) will lead to a reduction in conditions such as constipation, diabetes, obesity, coronary heart disease and others. In the 1970's Trowell had developed a definition of dietary fiber which evolved in 1976[3] to:

"Dietary fiber consists of the remnants of edible plant cells, polysaccharides, lignin, and associated substances resistant to digestion by the alimentary enzymes of humans."

This definition defines a macro constituent of foods which includes cellulose, hemicellulose, lignin, gums, modified celluloses, mucilages, oligosaccharides, pectins and associated minor substances such as waxes, cutin, and suberin.

On the basis of this definition,[3] appropriate methodology for the measurement of DF was developed by a consortium of researchers in Europe and USA. This led to AOAC Official Method 985.29 (the Prosky method),[4,5] and to subsequent modifications of this method, including AOAC Official Method 991.43[4] in which the buffers were changed. The aim of these methods was to give an accurate measurement of the content of total dietary fiber in plant products and food materials. More specifically, the methodology aimed at hydrolysing and removing starch and protein. Fats were removed by the solvents employed to recover the non-hydrolysed material. From the outset, it was realised that all protein was not hydrolysed, so each sample was then analysed in duplicate and residues recovered and weighed. One of these residues is analysed for ash content and the other for protein. These weights are subtracted from the average of the residue weights. It was also realised that, in the analytical procedure, starch also was not completely hydrolysed and removed. This in turn led to the discovery of so-called "resistant starch (RS)". The question then was, "*should RS be measured and added to the total dietary fiber value, or should it be analytically removed and ignored?*" Since RS escapes digestion in the human small intestine, the general consensus is that it should be accurately measured and included. Research in the 1990's showed that AOAC Official Method 991.43 underestimates RS, so alternative methods for the measurement of this component were developed and evaluated. While most of these new methods gave similar results for a range of RS containing samples, none of the methods survived the rigours of interlaboratory evaluation except that of McCleary *et al.*[6] (AOAC Method 2002.02), which also gave results in line with those obtained from ileostomy patients.

In the mid-1990's it was generally agreed that dietary fiber should also include non-digestible oligosaccharides (NDO), as these behaved physiologically as dietary fiber. Specific methods were developed for fructan [and fructo-oligosaccharides (FOS)] (AOAC Methods 997.08

and 999.03),[7,8] galacto-oligosaccharides (GOS) (AOAC Method 2001.03),[9] resistant maltodextrins (RMD) (AOAC Method 2001.03)[10] and Polydextrose® (AOAC Method 2000.11).[11] The development of these methodologies was very useful for ingredient developers, food manufacturers and analysts measuring the specific component. However, for those interested in measuring the total dietary fiber content of a material, a problem was introduced. For many of these specific carbohydrates (including RS, inulin and resistant maltodextrins) a portion of the component is also measured by AOAC Official method 985.29 and 991.43. Thus if the value for a component determined using the specific method is added to the total dietary fiber value, some of the component is double counted. This is clearly depicted in Figure 1.

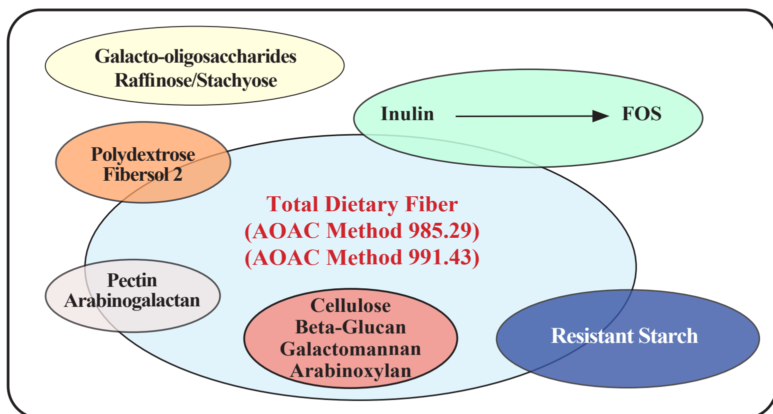

Figure 1. Components measured, and not measured, by AOAC Official Methods 985.29 and 991.43.

This problem of potential double counting led us to research and develop a procedure that allows the measurement of total dietary fiber, which includes RS and NDO. This integrated total dietary fiber (INTDF) procedure is depicted in Figure 2 and is modelled on AOAC Official Methods 2002.02 (resistant starch), 991.43 (total dietary fiber) and 2001.03 (resistant maltodextrins). The theory of this method is discussed in detail in McCleary (2007)[12] and has been successfully subjected to an interlaboratory evaluation through AOAC International (AOAC Method 2009.01).[13] A modified method for separately measuring insoluble dietary fiber (**IDF**), dietary fiber soluble in water but precipitated in 78% aqueous ethanol (**SDFP**), and dietary fiber soluble in water and not precipitated in 78% aqueous ethanol (**SDFS**) has also been successfully evaluated (AOAC Method 2011.25).[14] SDFP was previously termed high molecular weight soluble dietary fiber (**HMWSDF**) and SDFS was previously termed low molecular weight soluble dietary fiber (**LMWSDF**) and is also referred to as non-digestible oligosaccharides (**NDO**).

Several minor modifications to ensure complete measurement of FOS and complete removal of non-resistant maltodextrins are described

by McCleary *et al.* (2013),[15] as also are the problems in measurement of RS4 (starch granules chemically cross-linked with phosphate groups).

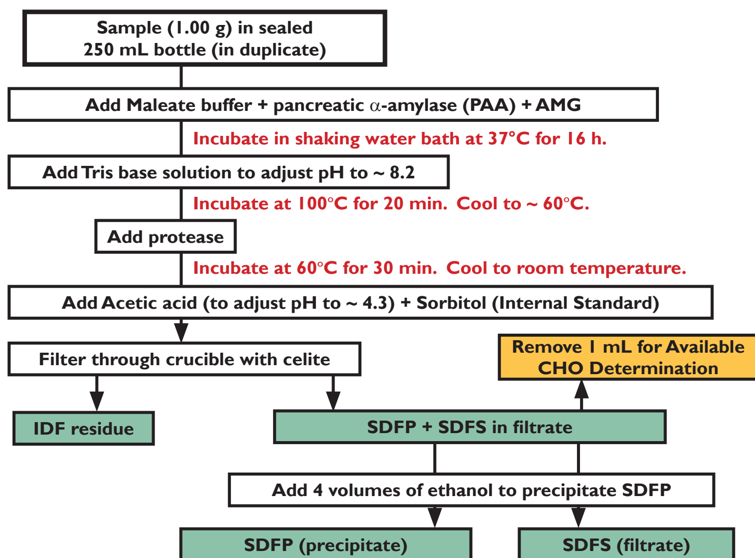

Figure 2. Principle of the Integrated (Codex compliant) Total Dietary Fiber assay procedure showing separate measurement of IDF, SDFP and SDFS.

In November 2008, the Codex Committee on Nutrition and Foods for Special Dietary Uses (CCNFSDU) established a definition for dietary fiber. This definition was accepted by the Codex Alimentarius Commission (CAC) in 2009 (FAO, 2009),[16] marking the achievement of international consensus:

"Dietary fiber consists of carbohydrate polymers with ten or more monomeric units, which are not hydrolyzed by the endogenous enzymes in the small intestine of humans and belong to the following categories: edible carbohydrate polymers naturally occurring in the food as consumed; carbohydrate polymers which have been obtained from food raw material by physical, enzymatic or chemical means and which have been shown to have a physiological effect of benefit to health as demonstrated by generally accepted scientific evidence to competent authorities, and; synthetic carbohydrate polymers which have been shown to have a physiological effect of benefit to health as demonstrated by generally accepted scientific evidence to competent authorities.

a) When derived from a plant origin, dietary fiber may include fractions of lignin and/or other compounds when associated with polysaccharides in the plant cell walls and if these compounds are quantified by the AOAC gravimetric analytical method for dietary fiber analysis: fractions of lignin and the other compounds (proteic fractions, phenolic compounds,

waxes, saponins, phytates, cutin, phytosterols, etc.) intimately "associated" with plant polysaccharides in the AOAC 991.43 method.

b) Decision on whether to include carbohydrates of 3 to 9 monomeric units should be left up to national authorities."

The Integrated TDF method described in this booklet, as originally published in 2007,[12] has been successfully subjected to interlaboratory evaluation through AOAC International (Methods 2009.01[13] and 2011.25[14]), and accepted by Codex Alimentarius as a Type I method (March, 2011).

SCOPE:

Applicable to all samples containing dietary fiber, including RS and NDO (also referred to as low molecular weight soluble dietary fiber; LMWSDF; SDFS), e.g. cereal grains, fruit and vegetables, cereal and fruit products and foods.

ASSAY KIT FOR INTEGRATED TDF:

Kits suitable for performing 100 assays of TDF (Integrated Procedure) are available from Megazyme. The kits contain the full assay method plus:

Bottle 1: Concentrated pancreatic α-amylase (**E-PANAA**); 4 g, 75,000 Ceralpha Units/g.
Stable for > 5 years when stored dry below -10°C.

Bottle 2: Amyloglucosidase (**E-AMGDF**) (20 mL, 3,300 Units/mL).
Stable for > 3 years at 4°C.

Bottle 3: Purified protease (**E-BSPRT**) (10 mL, 350 tyrosine units/mL).
Stable for > 3 years below -10°C.

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

Bottle 5: D-Sorbitol (approx. 12 g, dry).
Stable for > 3 years; store sealed at room temperature.

Celite 545®, acid washed, in 100 g or 500 g packages (cat. no. **G-CELITE**), Amberlite® FPA53 (OH^-) (cat. no. **G-AMBOH**) and Ambersep® 200 (H^+) (cat. no. **G-AMBH**) ion exchange resins are available separately from Megazyme.

AN INTEGRATED PROCEDURE FOR THE MEASUREMENT OF TOTAL DIETARY FIBER (INCLUDING RESISTANT STARCH AND NON-DIGESTIBLE OLIGOSACCHARIDES)

A. PRINCIPLE:

An integrated procedure (AOAC Method 2009.01 and 2011.25) is described for the measurement of total dietary fiber, including RS and SDFS (i.e. NDO) of DP $\geq$ 3. This method combines the key attributes of AOAC Official Methods of Analysis 2002.02, 985.29, 991.43 and 2001.03. Duplicate test portions are incubated with pancreatic α-amylase (PAA) and amyloglucosidase (AMG) for 16 h at 37°C in sealed 250 mL bottles in a shaking water bath (Figure 3, page 18) while mixing with sufficient vigour to maintain continuous suspension. Alternatively, a 2mag Mixdrive 15® submersible magnetic stirrer (www.2mag.de) can be used (Figure 4, page 18). During this step, non-resistant starch is solubilised and hydrolysed to D-glucose, maltose and traces of partially resistant maltodextrins by the combined action of the two enzymes (These partially resistant maltodextrins are subsequently hydrolysed before analysis of SDFS by HPLC).[15, 17] The reaction is terminated by adjustment of the pH to 8.2 and temporary heating. Protein in the sample is denatured and digested with protease. Specific dietary fiber fractions are measured as follows:

i. Insoluble dietary fiber (IDF), Higher Molecular Weight Soluble Dietary Fiber (SDFP) and Lower Molecular Weight Soluble Dietary Fiber (SDFS) determination. **(AOAC Method 2011.25).** 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 IDF and SDFP. The aqueous ethanol filtrate from the SDFP fraction is concentrated, desalted and analysed by HPLC for SDFS.

ii. Total High Molecular Weight Dietary Fiber (HMWDF) and SDFS determination. **(AOAC Method 2009.01).** 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, desalted, reconcentrated and analysed by HPLC for SDFS. **Alternatively, this fraction can more simply be deionised and analysed as described in K-RINTDF (AOAC Method 2017.16).**

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. NDO such as FOS and GOS are not hydrolysed, and the degree of hydrolysis of Polydextrose® and Fibersol-2® is in line with the information provided by the suppliers.

To ensure the presence of the appropriate enzyme activity, and absence of undesirable enzyme activity, the materials listed below (available in the kit, **K-TDFC**, from Megazyme) are analysed using the entire procedure. Each new lot of enzymes should be tested, as should enzymes that have not been tested for the previous 6 months.

Test Sample	Activity Tested	Sample Wt (g)	Expected Recovery (%)
Citrus pectin	Pectinase[c]	0.1	87[c]
β-Glucan (barley)	β-Glucanase[a]	0.1	95-100
Wheat starch	α-Amylase[b]	1.0	0-1
Casein	Protease[b]	0.3	0-2
High amylose starch[d]	α-Amylase	1.0	~ 48
Galactan (larch)	Pectinase[c]	0.1	~ 84[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 AOAC Method 991.43 even with no enzymes in the incubations.

d This material contains a high level of "enzyme resistant" starch. This value is higher than TDF (29.3%) with AOAC Method 991.43.

B. APPARATUS:

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).

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

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(r)]*, 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 11, page 22).

e. ***Rubber ring adaptors***.— For use to join crucibles with filtering flasks (Figure 11, page 22).

f. ***Vacuum source***.— Vacuum pump or aspirator with regulator capable of regulating vacuum (e.g. Edwards XDS 10; single-phase 115/230V; product code: A72601906).

g. ***Water bath(s)***.— Rotary motion (150 rpm), large-capacity (20-24 L) with covers; capable of maintaining temperature of 37 ± 1°C and 60 ± 1°C; (e.g. Grant® OLS 200 shaking incubation bath) (Figure 3, page 18). Alternatively, use a 2mag Mixdrive 15® submersible magnetic stirrer with a 30 x 7 mm stirrer bar, set at 170 rpm In a Megazyme water bath (cat. no. **D-TDFBTH**) (Figure 4, page 18).

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

i. ***Ovens***.— Two, mechanical convection, set at 103 ± 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 5.0 mL Combitip® (to dispense 0.1 and 0.3 mL aliquots of AMG and 0.1 mL of protease solution).
- with 25 mL Combitip® (to dispense 3 mL aliquots of 0.75 M Tris buffer solution and 4 mL aliquot of 2 M acetic acid).

n. ***Dispensers***.— To dispense (1) 15 ± 0.5 mL of 78% v/v EtOH (or IMS), 95% v/v EtOH and acetone, or (2) 40 ± 0.5 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 11, page 22).

r. ***Muffle furnace***.— 525 ± 5°C.

s. ***Polypropylene columns***.— Bio-Rad, Econo-Pac™ Disposable Chromatography Columns (cat. no. 732-1010) with an Alltech One-Way Stopcock (cat. no. 211524) (Figure 7, page 20).

t. ***Liquid Chromatograph (LC)***.— With oven to maintain a column temperature of 90°C and a 50 µL injection loop. System must separate maltose from maltotriose.

u. ***Guard column (or pre-column)***.— Waters Guard Pak® LC pre-column inserts (part no. WAT015209) or equivalent.

v. ***LC column***.— **Waters Sugar-Pak®** 6.5 x 300 mm column (part no. WAT085188) or equivalent. Mobile phase distilled water plus ethylene diamine tetraacetic acid; disodium calcium salt ($Na_2CaEDTA$) (50 mg/L) *[C(l)]*; flow rate 0.5 mL/min; column temp. 90°C; run time 30 min to assure column cleaned out.

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

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

y. ***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.

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

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

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

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

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

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

C. REAGENTS:

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

b. ***Ethanol (or IMS) 78% v/v***.— Place 821 mL 95% v/v ethanol (or IMS) into a 1 L volumetric flask. Dilute to volume with deionised water. Mix well. Check the level and if necessary add more deionised water to bring it back up to the 1 L mark.

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

d. ***Stock amyloglucosidase (AMG) solution***.— (**Bottle 2**, page 4) 3,300 Units/mL in 50% v/v glycerol – Solution is viscous; dispense using a positive displacement dispenser. (**Note:** One Unit of enzyme activity is the amount of enzyme required to release 1 micromole of D-glucose from soluble starch per minute at 40°C and pH 4.5). AMG solution should be essentially devoid of β-glucanase, β-xylanase and detectable levels of free D-glucose. Stable for > 5 years at 4°C or below -10°C.

e. ***Pancreatic α-amylase (50 Units/mL)/AMG (3.4 Units/mL)***.— Immediately before use, dissolve 0.20 g of purified porcine pancreatic α-amylase (75,000 Units/g; AOAC Method 2002.01) (**Bottle 1**, page 4) in 290 mL of sodium maleate buffer (50 mM, pH 6.0 plus 2 mM $CaCl_2$ and 0.02% sodium azide) *[C(h)]* and stir for 5 min. Add 0.3 mL of AMG *[C(d)]*. Stable for > 2 years below -10°C.

f. ***Protease (50 mg/mL; 350 Tyrosine Units/mL) in 50% v/v glycerol***.— (**Bottle 3**, page 4). Solution is viscous; dispense using a positive displacement dispenser. Protease must be devoid of α-amylase and essentially devoid of β-glucanase and β-xylanase. Use as supplied. Stable for > 3 years at 4°C.

g. ***Deionised water.***

h. ***Sodium maleate buffer***.— 50 mM, pH 6.0 plus 2 mM $CaCl_2$ and 0.02% sodium azide. Dissolve 11.6 g of maleic acid in 1600 mL of 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 ($CaCl_2.2H_2O$) and 0.4 g of sodium azide and adjust the volume to 2 L. Stable for > 1 year at 4°C.

> **NOTE:** Do not add the sodium azide until the pH has been adjusted. Acidification of sodium azide releases a poisonous gas. Handle sodium azide and maleic acid with caution only after reviewing SDS, using appropriate personal protective gear and laboratory hood.

i. ***Tris buffer solution, 0.75 M***.— Add 90.8 g of Tris buffer salt (Megazyme cat. no. **B-TRIS500**) to approx. 800 mL of deionised water (pH ~ 10.5) and dissolve. Adjust volume to 1 L. Stable for > 1 year at room temperature.

j. ***Acetic acid solution, 2 M***.— Add 115 mL of glacial acetic acid (Fluka 45731) to a 1 L volumetric flask. Dilute to 1 L with deionised water. Stable for > 1 year at room temperature.

k. ***HCl solution, 150 mM***.— Add 25 mL of conc. HCl (Sigma cat. no. 258148 - 2.5 L, 37% v/v, 12 M) to 1.9 L of distilled water and adjust to 2.0 L. Stable for > 3 years at room temperature.

l. ***Deionised water containing $Na_2CaEDTA$ (50 mg/L)***.— Weigh 50 mg of $Na_2CaEDTA$ into a 1 L Duran bottle and dissolve in 1 L distilled water. Prepare fresh weekly; filter through 0.45 mm filter *[B(z)]* before use.

m. ***Sodium azide solution (0.02% w/v)***.— Add 0.2 g of sodium azide to 1 L of deionised water and dissolve by stirring. Stable for > 2 years at room temperature.

> **NOTE:** Do not add sodium azide to solutions of low pH. Acidification of sodium azide releases a poisonous gas. Handle sodium azide with caution only after reviewing SDS, using appropriate personal protective gear and laboratory hood.

n. ***D-Glucose LC standards (5, 10, 20 mg/mL)***.— Accurately weigh 0.5, 1.0, and 2.0 g portions of high purity (> 99.5%) D-glucose (Sigma cat. no. G5767) and transfer to 3 separate 100 mL volumetric flasks. To each flask pipette 10 mL of internal standard *[C(o)]*. Bring to volume with 0.02% sodium azide solution *[C(m)]*. Transfer solutions to 100 mL Duran® bottles. Stable for 1 year at room temperature.

o. ***D-Sorbitol (Internal standard for SugarPak® column)***.— 100 mg/mL containing sodium azide (0.02% w/v). Weigh 10 g of analytical grade (> 99%) D-sorbitol (**Bottle 5**, page 4) into a 100 mL volumetric flask. Dissolve in 80 mL of 0.02% (w/v) sodium azide solution *[C(m)]* and adjust to volume with 0.02% sodium azide solution. Mix well. Stable for > 2 years at room temperature or stable for > 4 years below -10°C .

> **NOTE:** Handle sodium azide with caution only after reviewing SDS, using appropriate personal protective gear and laboratory hood.

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

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

r. ***Cleaning solution***.— Micro-90® (International Products Corp., USA, www.ipcol.com). Make a 2% solution with deionised water.

s. ***Mixed-bed ion exchange resins for each test portion***.—
(1) m-1. — approx. 4 g Amberlite® FPA53 (OH^-) resin (Rohm and Haas, France S.A.S.) (see also Megazyme **G-AMBOH**), ion exchange capacity 1.6 meq/mL (min) or equivalent (R-OH exchange capacity data supplied by manufacturer).
(2) m-2.— approx. 4 g Ambersep® 200 (H^+) resin or equivalent, (Rohm and Haas, France S.A.S.) (see also Megazyme **G-AMBH**), ion exchange capacity: 1.6 meq/mL (minimum).

Mix the two resins just prior to use and pack in column *[B(s), Bio-Rad disposable chromatography column]* for analysis of each test portion (see Figure 7, page 20). After mixing and packing, overlay the resin with a small layer (wad) of cotton wool and then wash the resin with 20 mL of deionised water.

t. ***Celite®***.— acid-washed, pre-washed (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 (**K-TDFC**) each time the enzyme lot changes or after the enzyme has been stored for more than 6 months.

F. ENZYME DIGESTION OF SAMPLES (AOAC Methods 2009.01 and 2011.25):

(1) Blanks

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

(2) Samples

(a) ***Weigh duplicate***.— 1.000 ± 0.005 g samples accurately into 250 mL Fisherbrand® soda glass, wide mouth bottles *[B(b)]*.

(b) ***Addition of Enzymes***.— Wet the sample with 1.0 mL of ethanol and add 40 mL of pancreatic α-amylase/AMG mixture *[C(e)]* 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 the springs in the shaker frame. Alternatively, use a 2mag Mixdrive 15® submersible magnetic stirrer *[B(g)]* with 7 x 30 mm stirrer bars (Figures 3 and 4, page 18).

(c) ***Incubation with pancreatic α-amylase/AMG***.— Incubate the reaction solutions at 37°C and 150 rpm in orbital motion in a shaking water bath *[B(g)]*; or at 170 rpm on a 2mag Mixdrive 15® submersible magnetic stirrer (to ensure complete suspension) for **exactly** 16 h (e.g. 5.00 pm to 9.00 am).

(d) ***Adjustment of pH to approx. 8.2 (pH 7.9-8.4), Inactivation of α-amylase and AMG***.— After 16 h, remove all sample bottles from the shaking water bath and immediately add 3.0 mL of 0.75 M Tris buffer solution *[C(i)]* to terminate the reaction. (At the same time, if only one shaker bath is available, increase the temperature of the shaking incubation bath to 60°C in readiness for the protease incubation step). Slightly loosen the caps of the sample bottles and immediately place the bottles in a water bath (non-shaking) at 95-100°C, and incubate for 20 min with occasional shaking (by hand). Using a thermometer, ensure that the final temperature of the bottle contents is > 90°C (checking of just one bottle is adequate).

(e) ***Cool***.— Remove all sample bottles from the hot water bath (use appropriate gloves) and cool to approx. 60°C.

(f) ***Protease treatment.***— Add 0.1 mL of protease solution *[C(f)]* with a positive displacement dispenser (solution is viscous). Incubate at 60°C for 30 min.

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

(h) ***Internal standard***.— Add 1.0 mL of D-sorbitol internal standard solution (100 mg/mL) *[C(o)]* to each bottle and mix well.

(i) ***Proceed to step [G(a)]*** for determination of **HMWDF/SDFS (AOAC Method 2009.01)** or to ***step [H(a)]*** for determination of **IDF/SDFP/SDFS (AOAC Method 2011.25)**.

G. DETERMINATION OF HMWDF (IDF plus SDFP): (AOAC Method 2009.01):

(a) ***Precipitation SDFP***.— Pre-heat the sample to 60°C and add 192 mL (measured at room temperature) of 95% (v/v) EtOH (or IMS) pre-heated to 60°C. Mix thoroughly and allow the precipitate to form at room temperature for 60 min.

(b) ***Filtration setup***.— Tare crucible containing Celite® {from *[B(c)], page 6*} 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 from wash bottle. Apply suction to crucible to draw Celite® onto fritted glass as an even mat (see Figure 11, page 22).

(c) ***Filtration***.— Using vacuum, filter precipitated enzyme digest *[G(a)]* through crucible. Using a wash bottle with 78% (v/v) EtOH (or IMS) *[C(b)]*, quantitatively transfer all remaining particles to crucible. Retain filtrate and washings 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), 95% (v/v) EtOH (or IMS) and acetone.

(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. Cool in desiccator and weigh to nearest 0.1 mg. Subtract crucible and Celite® weight to determine ash.

(h) ***Determination of HMWDF***.— Subtract ash and protein from average residue weight and proceed to ***step [J]*** for calculation of **HMWDF**.

H. DETERMINATION OF IDF and SDFP SEPARATELY (AOAC Method 2011.25):

IDF

(a) ***Filtration setup***.— Tare crucible containing Celite® {from *[B(c)], page 6*} 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 (see Figure 11, page 22).

(b) ***Filtration***.— Using vacuum, filter the enzyme digest from step *[F(h)]* 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) to dislodge all particles from the walls of the container. Transfer this suspension to the crucible. Wash the bottle with a further 10 mL of water at 60°C and again transfer to the crucible. Collect the combined filtrate and washings and adjust the volume to 70 mL and retain this for determination of ***SDFP [H(f)]*** and ***SDFS [I(a)].***

(c) ***Wash.***— Using a vacuum, wash the residue successively with two 15 mL portions of the following: 78% (v/v) EtOH (or IMS), 95% (v/v) EtOH (or IMS) and Acetone. Discard the washings.

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

(e) ***Cool crucibles in desiccators*** for approx. 1 h. Weigh crucible containing IDF residue and Celite® to nearest 0.1 mg. To obtain residue mass, subtract tare weight, i.e. weight of dried crucible and Celite®. Calculate IDF; ***step [J]***, as shown on page 17.

SDFP

(f) **Precipitation of SDFP**.— Pre-heat the filtrate of each sample (approx. 70 mL) to 60°C and add 280 mL (measured at room temperature) of 95% (v/v) EtOH (or IMS) *[C(a)]* pre-heated to 60°C and mix thoroughly. Allow the precipitate to form at room temperature for 60 min.

(g) **Recovery of SDFP and SDFS**.— Proceed according to ***step [G(b)]*** to ***[G(h)]*** on page 13.

(h) **For determination of SDFS**.— Proceed according to ***step [I(a)]*** to ***[I(f)]*** on pages 15-16.

I. DETERMINATION OF SDFS:

Note 1: ***Since the development and publication of the INTDF method, an improved procedure for measurement of TDF has been developed, namely, the Rapid Integrated TDF method (AOAC Method 2017.16; Megazyme cat no. K-RINTDF). We strongly recommend the use of this method to simplify analyses and obtain more physiologically relevant analyses.***

Note 2: ***Proper deionisation is an essential part of obtaining quality chromatographic data on SDFS***.— To obtain familiarity regarding the appearance of salt peaks in the SDFS chromatograms, dissolve 10 mg of sodium chloride in 9 mL of deionised water and add 1 mL of 100 mg/mL LC internal standard *[C(o)]* and proceed to step *[I(c)]* at "*Transfer the solution to a 10 mL disposable......*". To ensure the resins being used are of adequate deionising capacity, dissolve 10 mg of sodium chloride in 1 mL of deionised water. Add 1 mL of 100 mg/mL LC internal standard *[C(o)]*, and proceed to step *[I(b)]* at "*Transfer 2 mL of this solution to the top of.....*". The LC chromatogram of this solution should show no peaks in the time range corresponding to carbohydrates of DP3 or greater.

(a) ***Filtrate recovery and concentration***.— {Set aside the filtrate from one of the sample duplicates *[G(c)]* to use in case of spills or if duplicate SDFS data is desired}. Transfer one half of filtrate *[G(c)]* of the other sample duplicate to a 500 mL evaporator flask and evaporate to dryness under vacuum at 60°C.

(b) ***Deionisation of sample***.— Add 5 mL of deionised water to the evaporator flask and swirl the flask for approx. 2 min to dissolve the sample. Transfer the solution to a sealable polypropylene 20 mL container. Transfer 2 mL of this solution to the top of the Bio-Rad disposable column [B*(c)*] containing 4 g each of freshly prepared and thoroughly mixed, Amberlite FPA 53 (OH^-) [C*(s)*] and Ambersep 200 (H^+) [C*(s)*] (see Figure 7, page 22). Elute the column at a rate of 1.0 mL/min into a 100 mL Duran® bottle. When the sample has entered the resin, add 2 mL of distilled water to the resin and allow this to percolate in. Then add approx. 20 mL of deionised water to the top of the column and continue to elute at a rate of 1.0 mL/min. Transfer the eluate to a 250 mL round bottom rotary evaporator flask and evaporate to dryness under vacuum at 60°C. Add 2 mL of deionised water to the flask and redissolve the sugars by swirling the flask for approx. 2 min. Using a Pasteur pipette, transfer the solution to a polypropylene storage container.

(c) ***Preparation of samples for LC analyses***.— Transfer the solution to a 10 mL disposable syringe *[B(bb)]* and filter through a 0.45 µm filter *[B(y)]*. Use a 100 µL LC glass syringe *[B(cc)]* to fill the 50 µL injection loop on the LC *[B(t)]*. Perform this analysis in duplicate. Column: Waters Sugar-Pak® (6.5 x 300 mm). Solvent: distilled water containing Na_2Ca-EDTA (50 mg/L). Flow rate: 0.5 mL/min. Temperature: 90°C.

(d) ***Determine the response factor for D-glucose.***— Since D-glucose provides an LC refractive index (RI) response equivalent to the response factor for the non-digestible oligosaccharides that make up SDFS the LC is calibrated using D-glucose, and the response factor is used for determining the mass of SDFS. Use a 100 µL LC syringe to fill a 50 µL injection loop for each standard D-sorbitol/D-glucose solution. Inject in triplicate. Obtain the values for the peak areas of D-glucose and internal standard from the 3 chromatograms. The reciprocal of the slope obtained by comparing the ratio of peak area of D-glucose/peak area of D-sorbitol internal standard (y-axis) to the ratio of the mass of D-glucose/mass of D-sorbitol (x-axis) is the "response factor". Determine the average response factor (typically 0.97 for D-sorbitol).

Response factor (R_f) = (PA-IS) / (PA-Glu) x (Wt-Glu / Wt-IS)

where:

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

(e) ***Calibrate the area of chromatogram to be measured for LMWSDF.***— Use a 100 µL LC syringe *[B(cc)]*, to fill the 50 µL injection loop with retention time standard *[C(p)]*. Inject in duplicate. Determine demarcation point between DP 2 and DP 3 oligosaccharides (disaccharides maltose versus higher oligosaccharides) (see Figure 10, page 22).

(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.

NOTE: An in-line desalting procedure has been published.[17] This procedure is more convenient, but the disposable desalting cartridges are costly and relatively few samples can be desalted with one cartridge.

J. CALCULATIONS FOR HMWDF, IDF and SDFP:

Blank (B) determination (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 (mg) of protein and ash respectively, determined on first and second blank residues.

HMWDF, IDF or SDFP (mg/100 g):

$$= \frac{\frac{R_1 + R_2}{2} - P_B - P_A - B}{\frac{M_1 + M_2}{2}} \times 100$$

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.

HMWDF (%) = HMWDF (mg/100 g)/1000
IDF (%) = IDF (mg/100 g)/1000
SDFP (%) = SDFP (mg/100 g)/1000

K. CALCULATIONS FOR SDFS:

SDFS (mg/100 g)
= R_f x (Wt-IS, mg) x (PA-SDFS)/(PA-IS) x 100/M

where:

R_f is the response factor (see page 16).

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

PA-SDFS is the peak area of the SDFS.

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

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

L. CALCULATION OF INTEGRATED TDF:

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

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

Figure 3. Picture showing mixing of samples (in rotary motion) in a Grant® OLS 200 shaking incubation bath at 37°C.

Figure 4. Picture showing a 2mag Mixdrive 15® submersible magnetic stirrer in a custom made water bath (Megazyme water bath (cat. no. **D-TDFBTH**). This allows stirring of 15 samples at controlled speed (170 rpm) and 37°C.

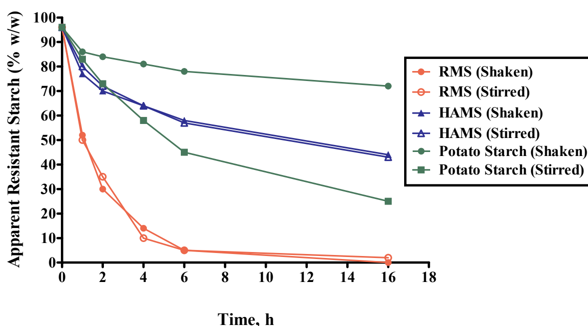

Figure 5. Rate of hydrolysis (and apparent resistant starch level) of regular maize starch (RMS), high amylose maize starch (HAMS) and native potato starch (PS) on incubation at 37°C with α-amylase/AMG in Duran® bottles either shaken in rotary motion at 150 rpm in a Grant® OLS 200 shaking incubation bath (Figure 3) or stirred at 170 rpm with α-amylase/AMG in Fisherbrand® bottles in a water bath at 37°C on a 2mag Mixdrive 15® submersible magnetic stirrer (Figure 4). **Note**: The values for HAMS and RMS are the same with both mixing and stirring. This was found for all samples studied except native potato starch, which has a fragile granule structure.

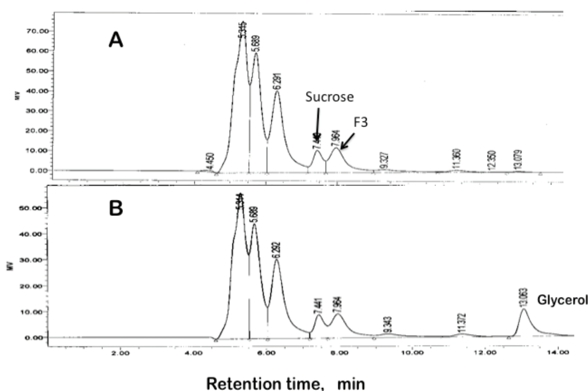

Figure 6. HPLC traces for Raftilose P-95® dissolved in water and analysed directly (A), compared with Raftilose P-95® recovered as SDFS after running through the Integrated TDF procedure (B). Column: Waters Sugar-Pak® (6.5 x 300 mm); solvent: distilled water containing Na_2Ca-EDTA (50 mg/L); flow rate: 0.5 mL/min; temp. 90°C. **Note:** No breakdown of the fructo-oligosaccharides. Also **note** that F3 (fructo-triose from hydrolysed inulin) elutes after sucrose (at the same point as lactose). For analysis of samples containing F3, a separate pre-treatment before HPLC is required (see Figure 10, page 22; also refer to the data sheet for **E-SUCRBG**).

Figure 7. Deionisation of samples with mixed bed resin [~ 4 g Amberlite® FPA53 (OH^-) and ~ 4 g Ambersep® 200 (H^+)] in Bio-Rad, Econo-Pac® Disposable Chromatography Columns connected to a Gilson Minipuls® Evolution pump.

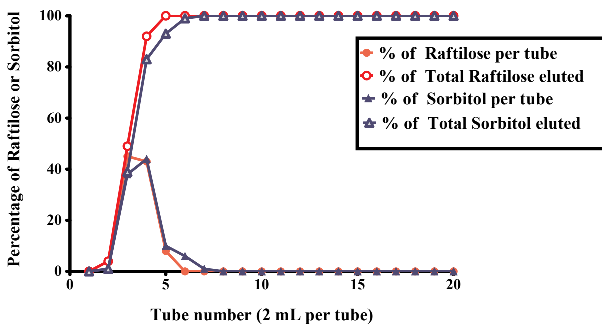

Figure 8. Elution of D-sorbitol and fructo-oligosaccharides from the mixed bed resin column. Note that the rate of elution of the D-sorbitol and fructo-oligosaccharides is the same and that essentially all carbohydrate is eluted with 20 mL of water.

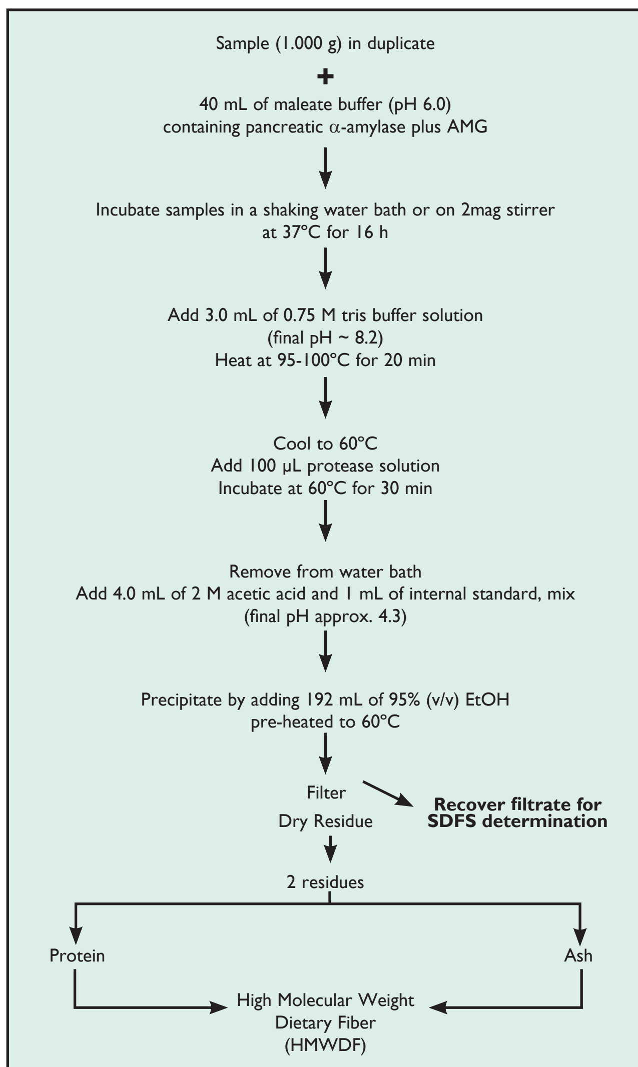

Figure 9. Analytical scheme for the determination of HMWDF (IDF + SDFP) and SDFS. If IDF and SDFP are to be separately determined, filter the reaction mixture before the addition of ethanol or IMS. The residue collected on the filter is IDF. Four volumes of ethanol or IMS are added to the filtrate to precipitate SDFP, which is recovered on a Celite® filter. The filtrate contains SDFS.

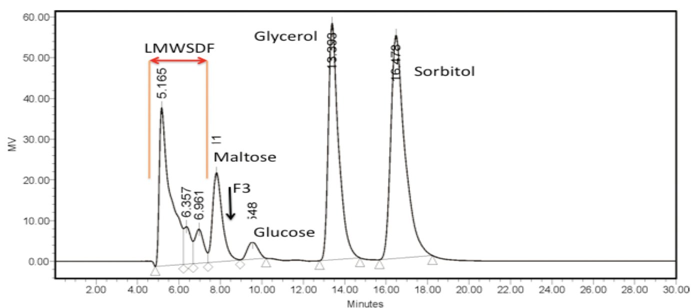

Figure 10. Chromatography of a mixture of maltodextrins, maltose, D-sorbitol and glycerol on a Waters Sugar-Pak® (6.5 x 300 mm); solvent: distilled water containing Na_2Ca-EDTA (50 mg/L); flow rate: 0.5 mL/min; temperature 90°C. The arrows show demarcation between DP 2 (maltose) and DP 3 (higher maltodextrins). **NOTE**: F3, the fructosyl-trisaccharide produced on hydrolysis of inulin by *endo*-inulinase or acid, elutes from the Sugar Pak® column after maltose (see arrow, F3, in above figure and see Figure 6). Samples containing this oligosaccharide, which is readily identified from the HPLC pattern, require pre-treatment with sucrase/maltase/ β-galactosidase (**see Frequently Asked Questions; FAQ**) or see recommended method in McCleary *et al.* (2013).[15]

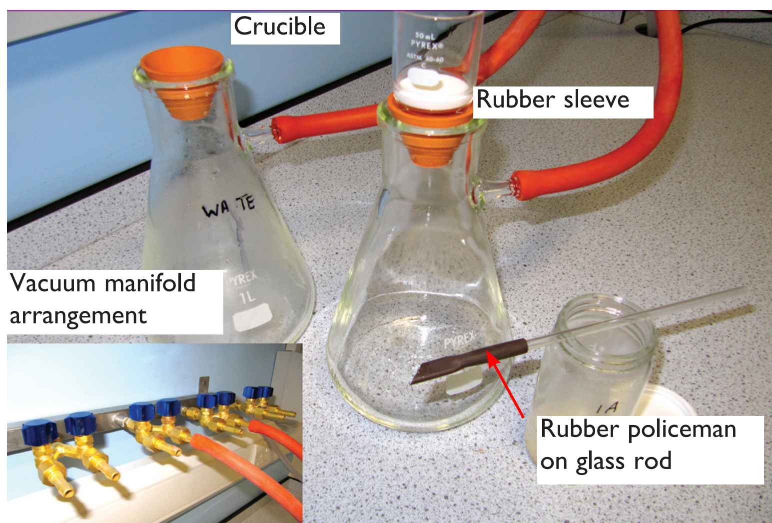

Figure 11. Büchner flask, crucible, rubber sleeve and vacuum manifold arrangement for filtration of dietary fiber samples. Picture also shows sample bottle and rubber policeman on a glass rod.

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Bray Business Park, Bray,
Co. Wicklow,
A98 YV29,
IRELAND.
Telephone: (353 1) 286 1220
Facsimile: (353 1) 286 1264)
Internet: www.megazyme.com
E-Mail: info@megazyme.com

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