

MAFF VALIDATED METHODS FOR THE ANALYSIS OF FOODSTUFFS

No. V2

DIETARY FIBRE (GLC)

Englyst Procedure for Determination of Dietary Fibre as Non-Starch Polysaccharides Plus Resistant Starch: Measurement of Constituent Sugars by Gas-Liquid Chromatography

Correspondence on this method may be sent to R. Wood, Statutory Methods (Chemistry and Microbiology) Department, Ministry of Agriculture, Fisheries and Food, Food Science Laboratory, Food Safety Directorate, Norwich Research Park, Colney, Norwich NR4 7UQ

1. Scope and field of Application

The method determines dietary fibre as non-starch polysaccharides (NSP) and as NSP plus resistant starch (RS).

2. Definition

The content of dietary fibre: the content of NSP or NSP plus RS as determined by the method specified.

3. Principle

The starch is gelatinised and then removed by enzymic digestion. The remaining polysaccharides are hydrolysed by sulphuric acid and the resulting individual neutral sugars are measured by gas-liquid chromatography (GLC) as their alditol acetate derivatives. Uronic acids are measured separately by a colorimetric procedure. Two alternative procedures are described for the dispersion of the starch thus enabling evaluation of the samples for dietary fibre as NSP alone and as NSP plus RS. The procedures are summarised in Appendix 1.

4. Reagents

High purity reagents are used throughout the method. Distilled water, or water of an equivalent purity, is to be used.

4.1 Acetone

4.2 Dimethyl sulphoxide

4.3 Benzoic acid, 50% saturated: prepare with saturated benzoic acid and water diluted 1 litre + 1 litre.

4.4 Sodium acetate buffer, 0.1 mol/l, pH 5.2.

4.4.1 Sodium acetate, 0.1 mol/l: prepare by dissolving 13.6 g sodium acetate trihydrate, $\text{CH}_3\text{COONa} \cdot 3\text{H}_2\text{O}$, and making up to 1 litre with benzoic acid (4.3).

4.4.2 Acetic acid, 0.1 mol/l

4.4.3 Calcium chloride, 1 mol/l

- 4.4.4 Sodium acetate buffer, 0.1 mol/l: adjust sodium acetate, 0.1 mol/l (4.4.1) to pH 5.2 with acetic acid, 0.1 mol/l (4.4.2). To stabilise and activate enzymes, add 4 ml of calcium chloride, 1 mol/l (4.4.3) to 1 litre of buffer.
- 4.5 α -Amylase, EC3.2.1.1: Pancrex V Capsules (approximately 9000 BP units α -amylase per capsule, Pains and Byrne Ltd.).
 - 4.5.1 α -Amylase solution, 2000 BP units/ml: empty 2 Pancrex V Capsules (4.5) into a centrifuge tube. Add 9 ml of water and disperse using a vortex-mixer. Centrifuge at 1500 g for 10 min. and use the supernatant as α -amylase solution. Prepare immediately before use.
- 4.6 Pullulanase, 100 units/ml: EC 3.2.1.41 (Boehringer 108944).
 - 4.6.1 Pullulanase solution, 1 unit/ml: dilute pullulanase (4.6) 1:100 (e.g. 0.010 ml made up to a final volume of 1 ml) with acetate buffer (4.4.4). Prepare immediately before use.
- 4.7 Ethanol, absolute.
- 4.8 Ethanol, 85% (V/V).
- 4.9 Sulphuric acid, 12 mol/l.
- 4.10 GLC Internal standard solution, 1 mg/ml: accurately weigh allose (dried to constant weight under reduce pressure with phosphorus pentoxide, P_2O_5) and make up the volume with benzoic acid solution (4.3).
- 4.11 Octan-2-ol
- 4.12 Ammonium hydroxide, 12 mol/l.
- 4.13 Ammonium hydroxide/sodium borohydride solution: ammonium hydroxide, 3 mol/l, containing 50 mg/ml sodium borohydride ($NaBH_4$).
- 4.14 1-Methylimidazole
- 4.15 Acetic acid, glacial.
- 4.16 Acetic anhydride
- 4.17 Potassium hydroxide, 7.5 mol/l.
- 4.18 Sodium chloride/boric acid solution: dissolve 2 g of sodium chloride, NaCl, and 3 g of boric acid, H_3BO_3 , in 100 ml of water.
- 4.19 Sulphuric acid, concentrated.
- 4.20 Dimethylphenol solution: dissolve 0.1 g of 3,5-dimethylphenol, $(CH_3)_2C_6H_3OH$, in 100 ml of glacial acetic acid (4.15).
- 4.21 Glucuronic acid, solid: for preparation of standards used in uronic acid determination (dried to constant weight under reduced pressure with phosphorus pentoxide, P_2O_5).
- 4.22 Standard sugar solutions, 1 mg/ml: use pure preparations of L-rhamnose, L-arabinose, D-xylose, D-mannose, D-galactose and D-glucose (dried to constant weight under reduced pressure with P_2O_5). Dissolve the sugars in, and make up the volume with benzoic acid solution(4.3).

4.23 Sulphuric acid, 2 mol/l.

5. Apparatus

5.1 Centrifuge

5.2 Centrifuge tubes: glass centrifuge tubes of 50-60 ml capacity, fitted with screw-tops.

5.3 Hot plate and stirrer. Place a beaker of water on the magnetic stirrer/hot plate and bring it to the required temperature. Use a beaker of sufficient capacity and height to contain the required number of tubes, e.g. 12. Place a layer of Scotch-Brite or similar material in the bottom of the beaker to prevent breakage. Cover the beaker, e.g. with tin foil, to aid boiling and ensure even temperature distribution.

5.4 Hot block

5.5 Magnetic stirrers, PTFE-coated, to fit centrifuge tubes (**5.2**).

5.6 Vortex-mixer

5.7 Water bath. This should be of such capacity that there is no significant change in temperature when a rack containing all the tubes, samples and standards is placed in it.

5.8 Spectrophotometer, capable of measurement at 400 and 450 nm.

5.9 Oven, capable of maintaining a temperature of $42 \pm 2^\circ\text{C}$ for 24 hr.

5.10 Gas-liquid chromatograph, fitted with flame ionisation detector and, preferably, peak area integrator and auto injector. The chromatographic column and conditions must be selected to separate and quantify alditol acetate derivatives of the individual sugars. The method described above has been developed based on the following conditions, but others are acceptable. A typical chromatogram is given in Appendix 2. When tailing from the solvent front increases, repack the first 5 cm of the column and recondition.

Chromatographic column : 2.1 m x 3 mm i.d., glass

Column packing : Supelcoport (100/120 mesh) coated with SP 2330

Injector temperature : 275°C

Column temperature : 215°C (isothermal)

Detector temperature : 275°C

Carrier gas : Nitrogen

Carrier gas flow-rate : 25 ml/min.

6. Procedure

6.1 Pre-treatment of sample

Foods are analysed without pre-treatment whenever possible. If there are problems in taking a representative sample, foods with a low water content may be milled and foods with a higher water content may be homogenised or freeze-dried and milled. No sample should be subjected to ball-milling for more than 2-3 min.

6.2 Test samples

Accurately weigh, to the nearest 0.1 mg, two portions (a) and (b) of the sample, each of between 100 and 500 mg (but containing more than 200 mg of dry matter and 50 mg of NSP, e.g. 200 mg flour, 100 mg bran) into a 50 ml screw-top centrifuge tube (5.2) and add a stirrer (5.4).

Portion (a) in tube (a) will be used to measure the total NSP content of the sample: portion (b) in tube (b) will be used to measure total NSP together with RS. These methods should be considered to be alternatives, so normally only tube (a) or tube (b) will be needed.

6.3 Fat extraction

Dry samples (i.e. 90-100% dry matter) with less than 5% fat may be analysed directly. Otherwise add 40 ml of acetone (4.1), mix for 30 min. using a magnetic stirrer, centrifuge and remove by aspiration as much of the supernatant as possible without disturbing the residue and dry it with stirring at $62.5 \pm 2.5^\circ\text{C}$ (see 6.4.2).

6.4 Dispersion and enzymic hydrolysis

6.4.1 Dispersion of the starch

Add 2 ml of DMSO (4.2) to tube (a) and mix for about 2 min. at room temperature, using the magnetic stirrer. Add 10 ml of acetate buffer (4.4.4) to tube (b). Cap and place tubes (a) and (b) in the beaker with boiling water (5.3) for 1 to 1.25 hr. timed from when re-boiling commences. Use the magnetic stirrer (note that in tube (a) gel formation may occur to such an extent that the stirrer is prevented from moving, but this will not affect the procedure).

Remove tube (a) from the beaker and immediately, without cooling, add 8 ml of acetate buffer (4.4.4), pre-equilibrated at $50 \pm 5^\circ\text{C}$, and vortex-mix. Then remove tube (b) from the beaker.

6.4.2 Enzymic hydrolysis of the starch

Note: All portions of the sample in their associated tubes are to be treated in the same manner in the procedures given in Section 6.4.2 through to Section 6.7 of the method. Reference to "a sample" or "a tube" is to be taken to include "all samples" or "all tubes" being analysed in this part of the method.

Leave the tubes at room temperature or in a water bath at 35°C , but only until contents have cooled to between 30 and 40°C and then immediately add 0.5 ml of α -amylase solution (4.5.1) followed by 0.1 ml of pullulanase solution (4.6.1) and vortex-mix. (NB: do not combine the enzyme solutions before addition).

Incubate the sample at $42 \pm 2^\circ\text{C}$ (5.7 or 5.9) for 16 to 18 hrs. Vortex-mix after the first hr.

After the enzyme treatment add 40 ml of absolute ethanol (4.7) to each tube, mix well by inversion and leave for 1 hr. at room temperature. Centrifuge at 1500 g for 10 min. for until a clear supernatant liquid is obtained.

Remove by aspiration as much of the supernatant as possible without disturbing the residue and discard it. Wash the residue twice with 85% ethanol (4.8), using 50 ml each time. Mix by inversion and then use a magnetic stirrer to form a suspension of the residue (about 5 to 10 min.), centrifuge until clear and remove the supernatant by aspiration as before.

Add 40 ml of acetone (4.1) to the washed residue, stir magnetically for 5 min. and then centrifuge at 1500 g for approximately 10 min. or until clear. Remove the supernatant by aspiration and discard it. Place the tube in a beaker of water at $62.5 \pm 2.5^\circ\text{C}$ on the hot plate stirrer (5.3) and mix the residue for a few min. until it appears dry. The beaker may be covered and the acetone vapour removed by a water pump.

6.5 Acid hydrolysis of the residue from enzymic digestion

Disperse the dried residue from section 6.4.2 in 2 ml of sulphuric acid, 12 mol/l (4.9) with vortex-mixing. Leave at $35 \pm 1^\circ\text{C}$ (5.6) for 1 to 1.25 hrs., with occasional mixing to disperse the cellulose. Rapidly add 22 ml of water and mix. Place in boiling water (5.3) for 2 to 2.25 hr. from re-boiling, stirring continuously. Cool, add 5 ml of gas-liquid chromatograph internal standard solution (4.10) and vortex mix.

6.6 Determination of neutral sugars by GLC

6.6.1 Preparation of alditol acetate derivatives.

Add 0.2 ml of ammonium hydroxide, 12 mol/l (4.12) to 1 ml of each hydrolysate (6.5). Test that the solution is alkaline, add a little more ammonium hydroxide if required, and then add 0.1 ml of a freshly prepared ammonium hydroxide/sodium borohydride solution (4.13) and 1 to 5 μl of octan-2-ol (4.11). Mix, leave for 1 hr. at 40°C in a hot block (5.3.1), add 0.1 ml of glacial acetic acid (4.15) and mix. To 0.5 ml of the acidified solution add 0.5 ml of 1-methylimidazole (4.14), 5 ml of acetic anhydride (4.16) and mix. Leave for 10 min. and then add 0.6 ml of ethanol (4.7) and mix. After 5 min. add 5 ml of water, mix and leave for a further 5 min. Place the tubes in a water bath at room temperature. Add 5 ml of potassium hydroxide, 7.5 mol/l (4.17) and a few min. later a further 5 ml of the potassium hydroxide. Cap each tube and mix by repeated inversion. Leave the tubes for 10 min. or until the separation into two phases is complete. Draw the top phase into a Pasteur or automatic pipette. If any of the lower phase is included, allow this to separate, then run it out before transferring the top phase alone to a small vial. Store at 5°C ; stable for 1 to 2 weeks.

Dilute each of the standard sugar solutions) with internal standard (4.10), 1 ml + 1ml. Then dilute these solutions with sulphuric acid, 2 mol/l (4.23), 1 ml + 1ml. Carry these mixtures through the procedure (6.6.1) above.

6.6.2 GLC

Carry out conventional GLC determination of the alditol acetate derivatives obtained from section 6.6.1 using the GLC column and conditions given in section 5.10; use 1-2 µl for injection onto the chromatograph.

6.7 Determination of uronic acids

Mix 0.3 ml of the supernatant obtained from section 6.5 (diluted if necessary to contain no more than 100 µg of uronic acids per ml: e.g. no dilution for flour, 1→5 for bran) with 0.3 ml of sodium chloride/boric acid solution (4.18) in 40-50 ml tubes. Add 5 ml of concentrated sulphuric acid (4.19) and vortex-mix. Place the tubes in a block (5.3.1) at 70°C. Leave for 40 ± 1 min. and cool to room temperature by placing in water.

When cool, add 0.2 ml of dimethylphenol solution (4.20) and vortex-mix immediately. Between 10 and 15 min. later, read the absorbance at 400 nm and 450 nm in a spectrophotometer (5.8) against a water reference.

Subtract the reading at 400 nm from that at 450 nm for each test sample. Plot the difference in absorbance obtained for glucuronic acid (4.21) standards over the range 25-125 µg/ml. Calculate or read sample concentrations from the graph.

7. COSHH

Analysts are reminded that appropriate hazard and risk assessments required by the Control of Substances Hazardous to Health Regulations, 1988 (See "Control of Substances Hazardous to Health - Approved Code of Practice, Control of Substances Hazardous to Health Regulations, 1988") must be made before using this method.

8. Expression of Results

All values are expressed as grams of polymers in 100 g of material taken for analysis.

8.1 Calculation of neutral sugars

Calculate the amount of each sugar in g/100 g by the following formula:

$$\text{sugar (g/100 g)} = \frac{W_s \times A_t \times 100}{A_s \times W_t}$$

where:

W_s is the weight (mg) of internal standard added (6.5).

A_t is the peak area for the test solution.

A_s is the peak area for the internal standard.

W_t is the weight (mg) of the test sample taken for analysis.

When using an auto-injector and a computing integrator, the internal standard (4.10) may be added as a constant percentage (e.g. 5) of the sample weight, thus allowing all calculations by computer as follows:

$$\text{sugar (g/100 g)} = \frac{A_t \times P_s}{A_s}$$

where:

P_s is the internal standard as weight percentage of the sample taken for analysis:

$$P_s = \frac{W_s \times 100}{W_t}$$

where:

W_s is the weight (mg) of the internal standard added (6.5).

W_t is the weight (mg) of the test sample taken for analysis.

8.2 Corrections

Experiments with mono- and poly-saccharides have shown that the hydrolysis and derivatisation procedures result in losses of approximately 10% of sugars. In order to express results as polymers or anhydro sugars, the GLC results should be decreased by approximately 10%. Because these two corrections are of approximately the same value, however, the GLC results are calculated and expressed directly as polymers or anhydro sugars.

There is incomplete (50%) hydrolysis and acetylation of any rhamnose. This is corrected for by applying a factor of $\times 2$ to the rhamnose values as determined experimentally. In practice, only traces of rhamnose are present in food products.

8.3 Calculation of non-starch polysaccharides

NSP = Rhamnose $\times 2$ + arabinose + xylose + mannose + galactose + glucose, all as measured in section 7.1 for test portion (a) + uronic acids as measured in section 6.7 for test portion (a).

8.4 Calculation of non-starch polysaccharides, together with resistant starch

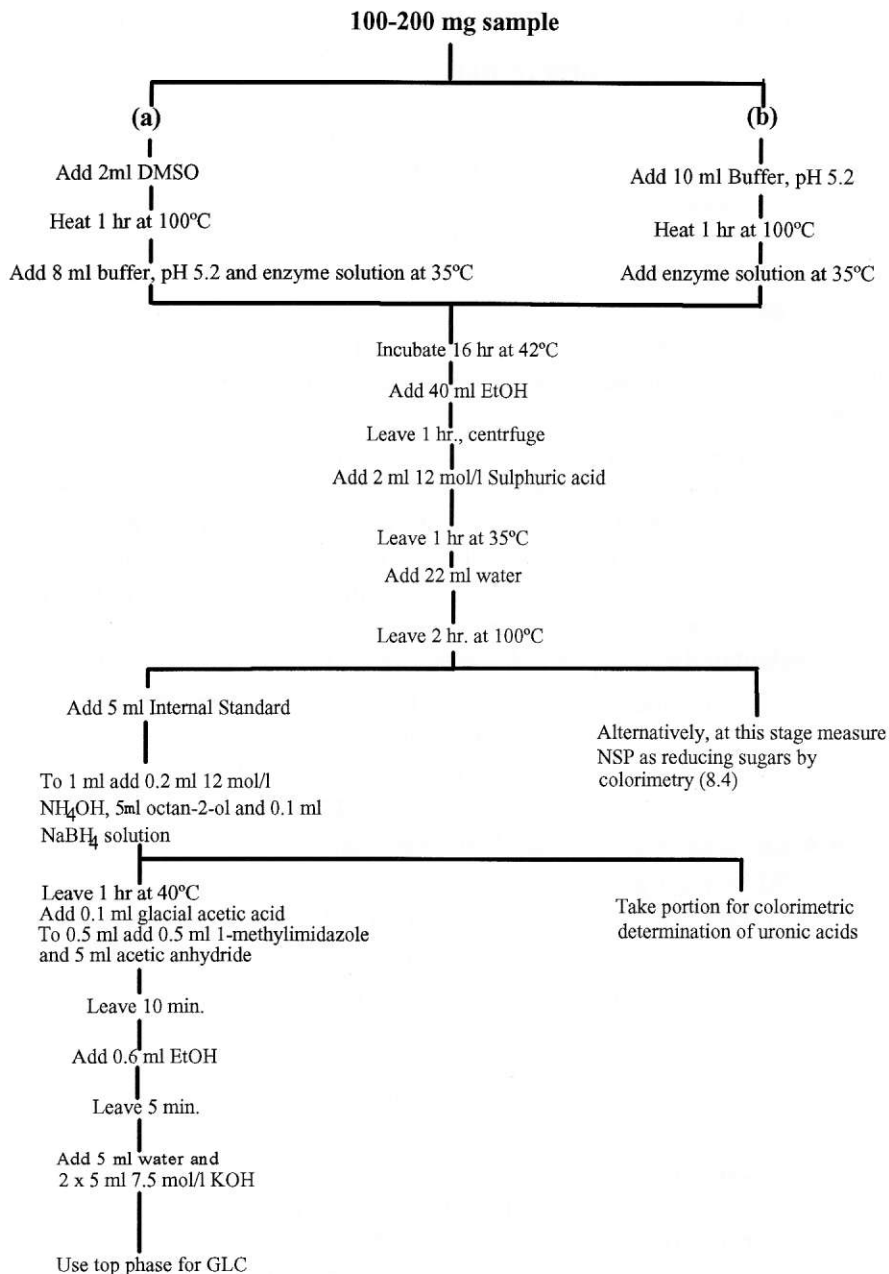
NSP + RS = Rhamnose $\times 2$ + arabinose + xylose + mannose + galactose + glucose, all as measured in section 7.1 for test portion (b) + uronic acids as measured in section 6.7 for test portion (b).

8.5 Calculation of resistant starch

RS = Glucose measured for test portion (b) minus glucose measured for test portion (a), as described in section 7.1. Note: RS levels calculated in this manner are unlikely to be precise (see Appendix 3).

APPENDIX 1

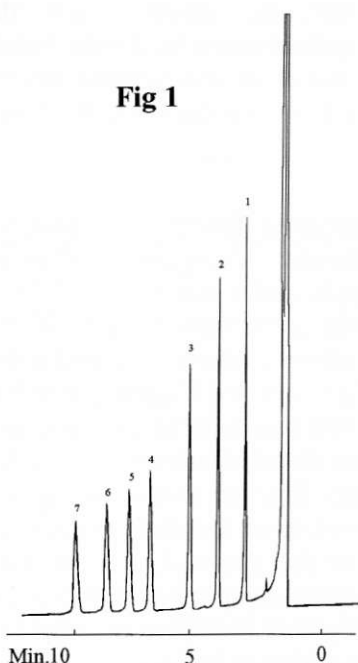
Schematic Diagram for Analysis of Dietary Fibre



APPENDIX 2

Typical Chromatogram of Standard Sugars

Fig 1



Standard sugar solution

1. Rhamnose;
2. Arabinose;
3. Xylose;
4. Allose (internal standard);
5. Mannose;
6. Galactose;
7. Glucose.

GLC column and conditions

3% SP-2330 on 100/120 Supelcoport in 2.10 m x 2 mm dia. glass column at 215°C; flow rate, 25 ml nitrogen per min; sample size, 1 µl.

9. References

- 9.1 HN Englyst, JH Cummings and R Wood. J. Assoc. Publ. Analysts, 1987, **25**, 73-110.
- 9.2 HN Englyst and JH Cummings. Analyst, 1984, **109**, 937-942.
- 9.3 HN Englyst and JH Cummings. J. Assoc. Off. Analyt. Chem., 1988, **71**, 808-814.
- 9.4 MAFF Validated Methods for the Analysis of Foodstuffs. No.V1; Dietary Fibre, Englyst Method: Colorimetric End-point Determination. London, 1990.

APPENDIX 3

Analytical Quality Control

General principles of analytical quality control are outlined in protocol V0 of the series.

A1 Repeatability

The absolute difference between two test results obtained under repeatability conditions should not be greater than the repeatability, r , deduced from the collaborative trial data summarised below (Tables 1 and 2). Although these do not show a consistent pattern among samples with different matrices and fibre contents, r should normally be taken as 2 g/100 g.

A2 Reproducibility

The absolute difference between two test results obtained under reproducibility conditions should not be greater than the reproducibility, R , deduced from the collaborative trial data below (Tables 1 and 2). Although these do not show a consistent pattern among samples with different matrices and fibre contents, R should normally be taken as 4 g/100 g.

A3 Trueness (Bias)

The trueness of the results cannot be estimated directly in the absence of pure analyte and of reference materials. Comparison of mean values from wholemeal and white breads and a mixture of the two indicates consistent recoveries during the collaborative trial. All the mean values of NSP and NSP plus RS observed during the trial agree quantitatively with those obtained by the simplified Englyst procedure (8.4); however, the full protocol described here may be regarded as a reference procedure since it gives more detailed information on the specific sugar composition of the dietary fibre. In this sense the full and simplified procedures are both accurate in the determination of dietary fibre as defined in Section 2 of this protocol, but observed values do not necessarily agree with results using other methods and other types of sample. In particular, they may not agree with the true level of polysaccharide that escapes assimilation by an animal during digestion, though they are designed to reflect this. It is recommended that analytical data should normally be based on NSP alone, since RS is usually a minor constituent, it depends on the processing history of the sample and its physiological significance is uncertain.

The determination of RS alone, by subtracting NSP from NSP + RS (calculated as in Section 7 of the protocol) introduces excessive variability and cannot be recommended. The method is not precise enough to be suitable for the estimation by difference of the small levels of RS usually encountered.

A4 Limit of Detection

This limit has not been established. The collaborative trial data suggests an accuracy which, if maintained at low fibre levels, corresponds to a lower limit of roughly 1 g/100 g for a duplicate determination.

A5 Statistical Data derived from the Results of Interlaboratory Tests

Participants in the collaborative trial at 19 laboratories each analysed a sample of wholemeal bread in duplicate as a pre-trial check. They then each analysed 14 samples once in the trial proper. These comprised 7 different samples (including the same wholemeal bread) in blind duplicate. The samples had been commercially prepared,

ground and homogenised; they were artificially coloured so as to appear identical.

Tables 1 and 2 summarise the statistical data; the dietary fibre levels were expressed as a percentage by mass of the sample on a dry weight basis, and $\bar{x}$ was the overall observed mean value.

TABLE 1

Statistical Analysis of the % Non-starch Polysaccharide in Various Matrices

Sample	$\bar{x}$	S_r	RSD_r	r	S_R	RSD_R	R
Wholemeal bread (pre-trial sample)	9.57	0.62	6.5	1.73	1.25	13.1	3.50
Wholemeal bread	9.33	0.60	6.5	1.69	1.37	14.7	3.83
Wholemeal/white bread (1:1)	6.66	0.79	11.8	2.20	1.08	16.1	3.01
White bread	2.78	0.31	11.0	0.86	0.58	20.9	1.63
High-bran bread	10.45	1.1	10.5	3.08	1.34	12.8	3.75
Rye bread	4.10	0.41	10.0	1.15	0.82	20.0	2.30
Cornflakes	0.81	0.23	28.7	0.65	0.41	51.1	1.16
Oat cereal	8.23	0.73	8.9	2.04	0.95	11.6	2.67

TABLE 2

Statistical Analysis of the % Non-starch Polysaccharide plus Resistant Starch in Various Matrices

Sample	$\bar{x}$	S_r	RSD_r	r	S_R	RSD_R	R
Wholemeal bread (pre-trial sample)	10.46	0.54	5.2	1.51	1.17	11.2	3.27
Wholemeal bread	10.69	0.69	6.5	1.94	1.93	18.0	5.40
Wholemeal/white bread (1:1)	7.75	1.06	13.7	2.97	1.41	18.2	3.95
White bread	3.93	0.24	6.2	0.68	0.77	19.5	2.15
High-bran bread	10.98	1.41	12.9	3.96	1.93	17.6	5.40
Rye bread	5.53	0.23	4.1	0.63	1.01	18.2	2.82
Cornflakes	4.18	0.72	8.5	2.02	0.88	21.1	2.46
Oat cereal	8.45	1.62	19.1	4.53	1.83	21.6	5.12

A6 Key to Tables 1 and 2

Symbol	Definition
$\bar{x}$	Overall mean value
S_r	The standard deviation of repeatability
RSD_r	The relative standard deviation of repeatability, expressed as a percentage of the mean (coefficient of variance of repeatability CV_r)
r	Repeatability
S_R	The standard deviation of reproducibility
RSD_R	The relative standard deviation of reproducibility, expressed as a percentage of the mean (coefficient of variance of reproducibility CV_R)
R	Reproducibility