J.Assoc.Publ.Analysts, 1996, 32, 1-38

'DIETARY FIBRE'

Measurement by the Englyst NSP Procedure

Measurement by the AOAC Prosky Procedure

Explanation of the Differences

Report of a Study Commissioned by the Ministry of Agriculture, Fisheries and Food

H.N. Englyst, M.E. Quigley, K.N. Englyst, L. Bravo and G.J. Hudson
Medical Research Council & University of Cambridge
Dunn Clinical Nutrition Centre, Hills Road, Cambridge, CB2 2DH, UK

September 1995

Contents

0004-5780/96 +38 $20.00

'DIETARY FIBRE'

1.0 Executive Summary

National dietary guidelines recommend an unfortified high-fibre diet rich in fruit, vegetables and wholemeal cereal products. A range of health benefits has been shown for this diet, which is a good source of anti-oxidants, vitamins and minerals, and is low in fat and energy. The unique characteristic of the plant foods that constitute a high-fibre diet is the presence of naturally occurring cell-wall material.

The principal aim of food labelling for dietary fibre is to guide the consumer in selecting the type of high-fibre diet that is recommended in the guidelines. Some measure of plant cell-wall material is thus required.

The purposes of the study were:

To analyse samples of the ten major food groups of the UK diet for 'dietary fibre' by the Englyst NSP procedure, which aims to measure plant cell-wall non-starch polysaccharides, and by the AOAC Prosky procedure, which aims to measure the sum of indigestible polysaccharides and lignin.

To analyse the AOAC Prosky residue in order to explain the differences between the values obtained by these two procedures and to examine the validity of the methods with respect to their stated aims.

To examine the consequences of using these methods for food labelling.

Analysis of the ten food groups:

For nine of the ten food groups, the AOAC Prosky values are higher than the NSP values, by 38% on average, ranging from 20 to 77%.

Detailed analysis of the gravimetric residue showed that the AOAC Prosky procedure underestimates the NSP content of foods, by 12% on average, ranging from 3% to 36%.

After measurement of ash, N × 6.25 and total polysaccharides, from 5 to 42% of the material in the AOAC Prosky residue remains unidentified. Klason lignin, which can contain Maillard reaction products and is a gross overestimation of lignin, ranged only from 0.4 to 4% of the sample for the ten food groups and does not account for the unidentified material in the AOAC Prosky residue.

A series of experiments showed:

Maillard reaction products can be produced by heating various combinations of carbohydrates and proteins and are measured by the AOAC Prosky procedure. Maillard reaction products are not corrected for as N × 6.25 in the AOAC Prosky procedure.

Some starch escapes digestion *in vivo* because it is inaccessible to enzymes (resistant starch types 1 and 2) but this starch is made available to enzymic hydrolysis *in vitro* by the milling and heating steps in the AOAC Prosky procedure and therefore not included in AOAC Prosky values. In contrast, starch that is readily digestible in foods that are normally eaten hot may retrograde during the cooling of samples for analysis to a form that is not digestible by the enzymes used in the AOAC Prosky procedure. Inclusion of this artefact leads to inflated AOAC Prosky values.

Fat, even at levels below that requiring extraction according to the procedure, inflates the AOAC Prosky values.

Organic acids are partially included in the AOAC Prosky values, and the proportion included is increased in the presence of calcium.

The stated aim of the AOAC Prosky procedure, to measure the sum of indigestible polysaccharides and lignin, is not achieved. The procedure does not measure inulin or all resistant starch; further, the procedure includes artefacts, such as Maillard reaction products, and highly variable amounts of unidentified material. As a result, the AOAC Prosky values represent an unspecified fraction of foods and are not suitable for food labelling because they cannot aid the consumer in choosing the recommended high-fibre diet.

Values for dietary fibre based on the measurement of plant cell-wall NSP aid the consumer in choosing the type of high-fibre diet recommended in the dietary guidelines and are therefore appropriate for food labelling.

2.0 Introduction

International trade requires standardised food labelling based on agreed methodology. Two main methods are in use for the measurement of dietary fibre; the Englyst NSP procedure (Englyst *et al.,* 1994), and the AOAC Prosky gravimetric method (Official Methods of Analysis, 1990). However, these methods include different components of the diet in the measurement and therefore give different values for identical foods.

The Englyst procedure aims to measure the plant cell-wall non-starch polysaccharides (NSP). This measurement reflects the plant cell-wall material that is characteristic of the high-fibre diet embodied in the dietary fibre hypothesis and in the national dietary guidelines.

The AOAC Prosky procedure aims to measure the sum of indigestible polysaccharides and lignin. This method therefore does not focus on plant cell-wall material, but seeks to include retrograded starch, which may be present in large amounts as the result of food processing. Higher values are therefore to be expected and considerably higher values are obtained by the AOAC Prosky than the Englyst procedure for many processed foods. Collaborative trials organised by the Ministry of Agriculture Fisheries and Food have suggested that the difference between the Englyst NSP and the AOAC Prosky gravimetric values are much too large to be explained by the inclusion of starch and lignin in the AOAC Prosky residue (Wood *et al.,* 1993).

The study described in this Report was undertaken to compare the results of analysis of ten samples representing the major food groups in the UK diet by the AOAC Prosky procedure and the Englyst NSP procedure.

The Englyst procedure measures NSP as the sum of chemically identified component sugars; the AOAC Prosky gravimetric procedure measures the weight of a residue, corrected for ash and crude protein content.

The primary objective was to establish the magnitude of the differences in the values obtained by the chemical and gravimetric approaches. Secondary objectives were to characterize the differences in the material measured by the two methods and to consider whether the two procedures meet their stated aims.

Food labelling for Fibre must serve as a guide for the consumer in the choice of the high-fibre diet recommended in the dietary guidelines. The results of this and related studies are taken into account in examining the consequences of using either of these methods for Fibre labelling.

3.0 Methods

(i) Primary study

The bulked samples were analysed by:

(1) The AOAC Prosky procedure (Official Methods of Analysis, 1990).

The AOAC Prosky procedure was followed using the Kit supplied by Sigma Chemical Co., which includes all the necessary enzymes and Celite as a filtering aid.

Reference materials may be purchased from Sigma separately as an Assay Control Kit.

(2) The NSP procedure (Englyst *et al.*, 1994).

The NSP procedure with the gas-liquid chromatography end-point was followed using the Kit supplied by Novo Nordisk, which includes all the necessary enzymes, a stock sugar mixture, an internal standard for GLC, and reference material.

(ii) Secondary study

(1) After filtration following the AOAC Prosky procedure, the residues were dispersed, the total polysaccharides were hydrolysed and measured according to Englyst *et al.* (1994), the starch content was measured according to Englyst *et al.* (1992a) and the Klason lignin content was measured as the residue remaining after hydrolysis of the polysaccharides.

(2) To study the mechanisms whereby the AOAC Prosky values are increased by food processing, combinations of amino acids, proteins, sugars and starch were heated before analysis by the AOAC Prosky procedure.

(3) To investigate the inclusion of starch retrograded during sample treatment as an artefact in the AOAC Prosky procedure, freshly cooked starchy foods were analysed immediately, and after cooling and freeze-drying of the analytical samples.

(4) The inclusion of organic acids in the AOAC Prosky procedure was studied by measuring the recovery of these in the residue both in the presence and absence of calcium.

4.0 Samples

Samples of ten major food groups were collected by MAFF in 1994 at six locations in the UK:

Berwick-upon-Tweed, Manchester, Mansfield, Newton Abbott, Streatham (London) and Swansea.

The samples were collected, frozen and transported to Cambridge, where they were freeze-dried and milled. Mixed bulked samples for each food group were prepared by mixing equal weights of the freeze-dried material from each collection centre.

The bulked samples were milled to pass a 0.5mm mesh; the Nuts sample was too fatty and the Fruit products sample was too sticky to pass the mesh and these samples were thoroughly mixed.

The food groups are:

Bread; Other cereals; Meat products; Green vegetables; Potatoes; Other vegetables; Canned vegetables; Fresh fruit; Fruit products; Nuts.

5.0 Analysis of the Ten Food Groups

The results (average and individual analytical results; and see Appendix I) of the analysis of the ten food groups by the AOAC Prosky procedure and by the NSP procedure are given in Table 1 and illustrated by Figure 1. With the exception of the Meat products sample, the AOAC Prosky

values are considerably higher than the NSP values, on average by 38%, with a range of differences from 20% (Green vegetables) to 77% (Other vegetables).

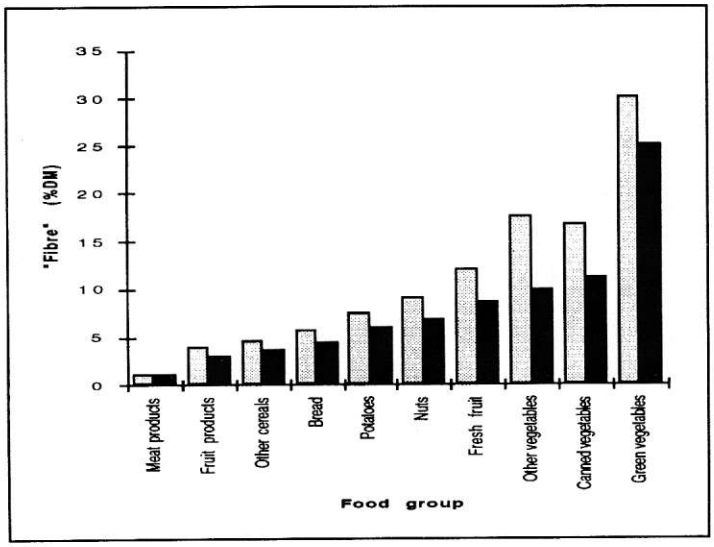
Table 1

Analysis of the ten food group samples for dietary fibre

Food group	Englyst (% DM)	AOAC Prosky (% DM)	Difference (% DM)
Bread	4.5 (4.3, 4.7)	5.9 (5.7, 6.1)	1.4
Other cereals	3.8 (3.7, 3.9)	4.8 (4.7, 4.9)	1.0
Meat products	1.1 (1.0, 1.2)	1.1 (1.1, 1.1)	0
Green vegetables	25.2 (24.3, 26.1)	30.2 (29.8, 30.6)	5.0
Potatoes	6.1 (6.0, 6.2)	7.6 (7.4, 7.8)	1.5
Other vegetables	10.0 (9.8, 10.2)	17.7 (17.6, 17.8)	7.7
Canned vegetables	11.3 (11.2, 11.4)	16.9 (16.3, 17.5)	5.6
Fresh fruit	8.7 (8.6,8.8)	12.1 (11.9, 12.3)	3.4
Fruit products	3.1 (3.1, 3.1)	4.0 (3.7, 4.3)	0.9
Nuts	6.9 (6.7, 7.1)	9.2 (8.9, 9.5)	2.3

Average (and individual) values are shown.

Figure 1



Average values for the ten food groups obtained by the AOAC Prosky procedure (light bars) and by the NSP procedure (dark bars).

The analytical values were converted to fresh matter and used with intake values to calculate the amounts of fibre provided in the UK diet as measured by the AOAC Prosky procedure and as measured by the NSP procedure. The results are given in Table 2 and illustrated by Figure 2. Total intake calculated using the AOAC Prosky values is 34% higher than that obtained using the NSP values. Equal intakes are obtained for the Meat products group and only small differences for the Fruit products and Nuts groups. However, the intakes calculated with the AOAC Prosky values for the other seven food groups are, on average, 37% higher than those calculated using the NSP values, ranging from 20% (Green vegetables) to 85% (Other vegetables) higher for single food groups.

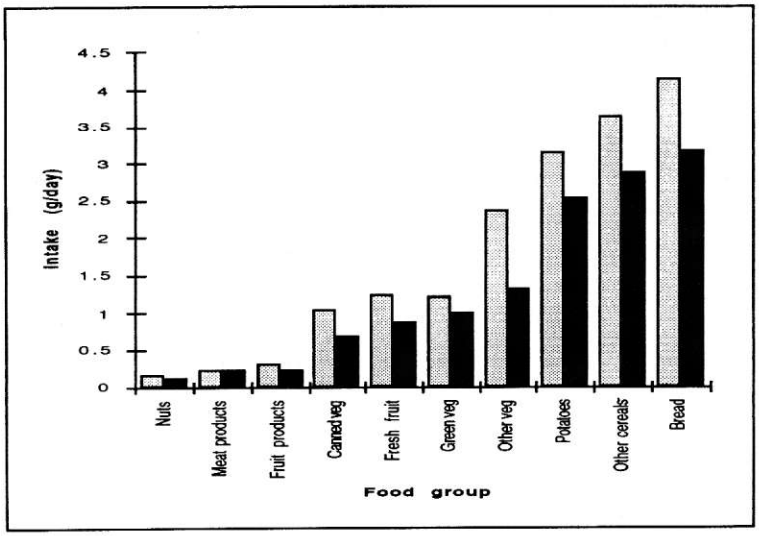
Table 2

Calculated intakes of 'fibre' using AOAC Prosky and NSP values

Food group	Food intake (g/day)	'Fibre' intake (g/day) AOAC	NSP	Diff.*
Bread	110	4.1	3.2	0.9
Other cereals	90	3.6	2.9	0.7
Meat products	44	0.2	0.2	0
Green vegetables	37	1.2	1.0	0.2
Potatoes	133	3.2	2.5	0.7
Other vegetables	73	2.4	1.3	1.1
Canned vegetables	35	1.0	0.7	0.3
Fresh fruit	65	1.2	0.9	0.3
Fruit products	43	0.3	0.2	0.1
Nuts	2	0.2	0.1	0.1
Total	632	17.4	13.0	4.4

*Calculated as AOAC – NSP for each food group.

Figure 2



The calculated intakes of 'fibre' from the ten food groups using the values measured by the AOAC Prosky procedure (light bars) and those measured by the NSP procedure (dark bars).

6.0 The AOAC Prosky Procedure

6.1 Stated aims

The stated aim of the AOAC Prosky method (Official Methods of Analysis, 1990) is to measure indigestible polysaccharides and lignin.

6.2 Principle

Four subsamples of dried foods, fat-extracted if containing more than 10% fat, are treated with enzymes (protease, α-amylase and amyloglucosidase) to remove protein and starch. Soluble material is precipitated in ethanol; the total residue is filtered, washed with ethanol and acetone, dried and weighed. Two subsamples are analysed for nitrogen; the others are incinerated at 525°C to determine total ash. An enzyme blank is used in the procedure. The calculation is:

$$\%DF = (R_S - P_S - A_S - B)/W_S$$

where R_S, P_S, A_S and W_S are average sample residue weight, average sample 'protein' weight, average sample ash weight and average sample weight, respectively. B is the blank value, calculated as:

$$B = R_B - P_B - A_B$$

Where R_B, P_B and A_B are average blank residue weight, average blank 'protein' weight and average blank ash weight, respectively, where all weights are in mg.

Total nitrogen is measured by the Kjeldahl procedure and protein is calculated as N × 6.25, except when some other factor is known to be more appropriate (6.25 is chosen as correcting for the average nitrogen content of food proteins but when the identity of the protein(s) is known it may be possible to use a more accurate value; e.g. 6.38 for bovine milk). If required, soluble dietary fibre can be obtained as described for the AOAC Prosky procedure (Official Methods of Analysis, 1990).

6.3 Are the stated aims achieved ?

We have measured the ash and total nitrogen in the residue as part of the AOAC Prosky procedure (see Appendix I for the values). The AOAC Prosky values are corrected within the method for ash and 'protein'

(N × 6.25) and so the remainder should be polysaccharides and lignin. We have measured the total polysaccharides and the total starch in the residue and calculated NSP in the residue as the difference; the results are given in Table 3. These calculated NSP values for the residue are slightly lower (12% on average, range 3 to 36%) than those obtained for NSP by direct analysis (Table 1), suggesting incomplete recovery of NSP by the AOAC Prosky procedure, in agreement with previous observations (average 11%, range 3 to 50%, see Appendix II). There is a specific problem with inulin, an indigestible polysaccharide that is soluble in 78% ethanol and therefore not included in AOAC Prosky values.

Table 3

The starch and NSP content of the AOAC Prosky residue and the Englyst NSP values

Food group	Starch in the AOAC Prosky residue *	NSP in the AOAC Prosky residue **	Englyst NSP
Bread	1.0 (0.8, 1.1)	4.1	4.5
Other cereals	0.3 (0.3, 0.2)	3.5	3.8
Meat products	0.3 (0.2, 0.3)	0.7	1.1
Green vegetables	1.5 (1.3, 1.9)	24.1	25.2
Potatoes	1.8 (1.5, 2.0)	5.4	6.1
Other vegetables	1.0 (0.8, 1.1)	9.2	10.0
Canned vegetables	2.3 (2.5, 2.0)	10.7	11.3
Fresh fruit	0.2 (0.1, 0.2)	8.4	8.7
Fruit products	0.1 (0.1, trace)	2.4	3.1
Nuts	0.4 (0.3, 0.4)	6.3	6.9

All the values are expressed in units of g/100g DM of the sample for ease of comparison both within this Table and with other Tables of results.

* Average and individual values.

** Calculated as the difference between total polysaccharides (see Table 4) and starch measured in the AOAC Prosky residue.

The difference between the AOAC Prosky values (after correction for ash and protein) and the value for total polysaccharide could be taken as an estimate of lignin if the AOAC Prosky procedure has achieved its aims. It is obvious from

the values in the Unidentified material column of Table 4 (and see Appendix II) that the residue must contain material outside the stated aim of the method; with the exception of the Meat products, the values are simply too large to be accounted for as lignin.

Table 4

Polysaccharides and unidentified material in the AOAC Prosky analytical residue for the ten food groups

Food group	AOAC Prosky 'DF' (g/100g DM)	Total polycachharides in the residue (g/100g DM)*	Unidentified material (g/100g DM)	(% 'DF')
Bread	5.9	5.1 (5.0, 5.2)	0.8	14
Other cereals	4.8	3.8 (3.8, 3.8)	1.0	21
Meat products	1.1	1.0 (1.0, 1.0)	0.1	9
Green vegetables	30.2	25.6 (25.5, 25.6)	4.6	15
Potatoes	7.6	7.2 (7.1,7.2)	0.4	5
Other vegetables	17.7	10.2 (10.2, 10.2)	7.5	42
Canned vegetables	16.9	12.9 (12.9, 12.9)	4.0	24
Fresh fruit	12.1	8.6 (8.5, 8.7)	3.5	29
Fruit products	4.0	2.5 (2.4, 2.6)	1.5	38
Nuts	9.2	6.7 (6.6, 6.8)	2.5	27

*Average and individual values.

The effects of cooking foods on the values obtained by the AOAC Prosky procedure (Table 5) can be quite marked, as shown by Prosky & DeVries (1992). We demonstrate that these increases in the AOAC Prosky values may be partly due to the inclusion of Maillard reaction products (MRPs) and partly to the retrogradation of starch during sample pretreatment for analysis.

Table 5

Effect of cooking on AOAC Prosky values

	AOAC Prosky 'DF' (% DM)			
Treatment	Brown rice	Farina	Noodles	Spaghetti
Raw	3.9	3.0	3.0	2.7
Cooked	6.3	9.8	7.0	4.5

Calculated from Prosky & DeVries (1992).

6.4 Artefacts in the AOAC Prosky procedure

In order to understand what could lead to these falsely high values, we investigated the mechanisms by which artefacts can be included in the AOAC Prosky residue to yield AOAC Prosky values that are greater than the sum of polysaccharides and lignin.

6.5 Formation of Maillard reaction products that are included in the AOAC Prosky procedure

The heating of mixtures of amino acids and reducing sugars leads to the formation of yellow-brown pigments. A vast range of compounds are so formed and these are collectively termed Maillard reaction products (MRPs) after their discoverer (Maillard, 1912). Some of the MRPs are aromatic compounds that enhance the flavour and taste of foods but many have been identified as potentially mutagenic/carcinogenic (Weisburger, 1994). We show here that MRPs formed upon heating carbohydrates and amino acid/protein mixtures under various conditions are included as artefacts in the AOAC Prosky values.

6.6.0 Experimental studies

6.6.1 Effect of heating a mixture of glucose and lysine

Samples (5ml) of a solution of 0.64M glucose, 0.32 M lysine were heated at 100° C in stoppered tubes for various lengths of time, up to 24 hours, before analysis by the AOAC Prosky procedure. The AOAC Prosky values are given in Table 6.

Table 6

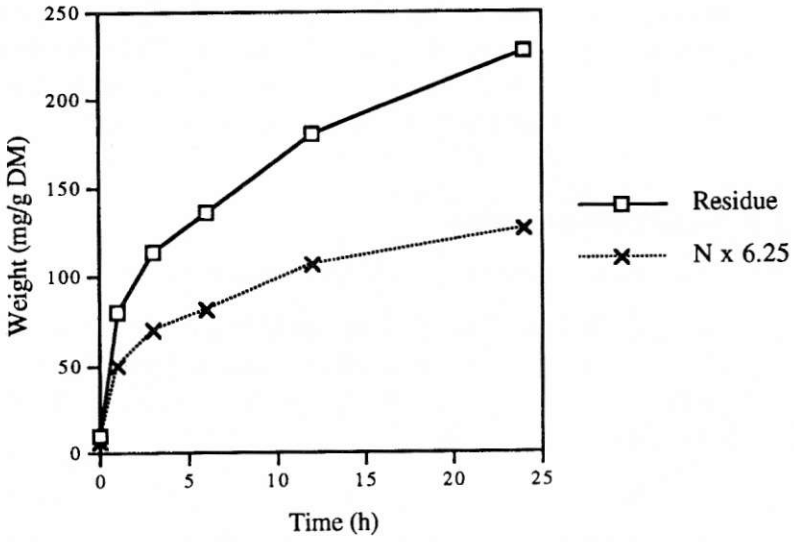
Effect of heating glucose and lysine together at 100° C

Time @ 100° C (hours)	AOAC Prosky 'DF' (% DM)
0	0.5
1	3.9
3	5.1
6	7
12	10.6
24	14.4

Glucose was not released by acid hydrolysis of the AOAC Prosky residue using the conditions described for the Englyst procedure, indicating that the residue does not contain synthesised glucose polymers, and ash values did not change significantly (data not shown).

Figure 3 shows the increase in the uncorrected residue weight (open squares) with time. It is clear that although the N × 6.25 values (crosses) also increase with time of heating, the magnitude of the increase does not account for the increase in the residue weight, because the nitrogen content of MRPs is different from that of protein.

Figure 3



Increase in AOAC Prosky uncorrected residue weight and N × 6.25 content with time of heating a mixture of glucose and lysine.

6.6.2 Effect of heating glucose with glycine, with albumin, and with casein

Mixtures (1:1, w/w) of glucose with glycine, egg albumin or bovine casein were heated under pressure (autoclave, 15psi) for one hour before analysis by the AOAC Prosky procedure and the results are shown in Table 7. As was seen for the heated mixture of glucose and lysine

(Table 6), there are substantial increases in the AOAC Prosky values after heating glucose in the presence of protein.

Table 7

Effect of heating glucose (Glc) with glycine, albumin or casein under pressure (autoclaving for 1 hour)

Sample	Heated	AOAC Prosky 'DF' (% DM)	Residue weight* (mg/g DM)	N × 6.25 in residue (mg/g DM)
Glc/Glycine	No	<0.1	9	8
	Yes	8.4	206	101
Glc/Albumin	No	0.7	136	103
	Yes	4.1	160	105
Glc/Casein	No	0.2	53	45
	Yes	2.5	110	78

*Residue weight before any correction.

6.6.3 Effect of heating albumin with sucrose and with starch

Mixtures (1:1, w/w) of egg albumin with sucrose, and with soluble potato starch (BDH) were heated at 200° C for various lengths of time before analysis by the AOAC Prosky procedure and the results are shown in Table 8. These results extend the study to demonstrate increases in the AOAC Prosky values as the result of the interaction of protein and starch upon heating.

Table 8

Effect of heating albumin and carbohydrate at 200° C

Sample	Time of heating (min)	AOAC Prosky 'DF' (% DM)	Residue weight* (mg/g DM)	N × 6.25 in residue (mg/g DM)
Albumin/sucrose	0	1.0	90	70
	15	15.3	547	376
Albumin/starch	0	1.0	111	80
	60	6.5	482	390

*Residue weight before any correction.

6.6.4 Effect of heating sucrose with a mixture of protein and starch

A mixture of 45g of sucrose, 60g of egg-white, 25g of white wheat flour and 25g of potato flour was heated at 200° C for seven minutes before analysis by the AOAC Prosky procedure and the results are shown in Table 9. The increase in AOAC Prosky values is shown here for a mixture of typical food products after only brief heating.

Table 9

Effect of heating sucrose with egg, wheat flour and potato flour

Time of heating (min)	Prosky AOAC 'DF' (% DM)	Residue weight (mg/g DM)	N × 6.25 in residue (mg/g DM)
0	1.3	33	15
7	3.2	109	66

*Residue weight before any correction.

It has been suggested that the correction for protein (subtraction of N × 6.25 values) in the calculation of the Prosky AOAC values provides adequate correction for the presence of both protein and Maillard reaction products. Tables 6 to 9 show the increase obtained in the Prosky AOAC values that results upon heating simple mixtures of carbohydrate and protein. The residue weights shown in Tables 6 to 9 are before subtraction of the ash values, which do not vary significantly with treatment, or subtraction of the N × 6.25 values.

As illustrated by Figure 3, the N × 6.25 values do increase after heating, but the magnitude of the increase does not account for the increase in the residue values. Any Maillard reaction products included in the residue will contribute to the Prosky AOAC values, as shown in the Unidentified material column of Table 4 (and see Appendix II).

6.6.5 Inclusion of starch retrograded during sample preparation as an artefact in the Prosky AOAC residue

Table 10 shows the Prosky AOAC values for rice, potato, pasta and haricot beans analysed: raw; freshly cooked; and after cooling and freeze-drying, which is the normal pretreatment for analytical samples in the Prosky AOAC procedure. The values for the raw and the freshly cooked foods are very similar; however, when the samples were cooled and freeze-dried for analysis, the Prosky AOAC values increased substantially (from 22% for haricot beans to 67% for rice) relative to those obtained for the freshly cooked samples.

This increase in Prosky AOAC values is due to the inclusion of starch that has retrograded to an enzyme-resistant form during the treatment of the sample for analysis. Even when there is no resistant starch in a food as eaten hot, the Prosky AOAC procedure can include starch that has retrograded during the cooling of samples for analysis. Many of the available Prosky AOAC values for cooked starchy foods are apparently artificially inflated by including starch that is normally readily digested.

Table 10

Inclusion of starch retrograded during sample preparation as an artefact in the Prosky AOAC residue

	Prosky AOAC 'DF' (% DM)			
	Rice	Potato	Pasta	Haricot beans
Raw	1.5	7.4	4.1	16.6
Freshly cooked	1.5	7.2	4.1	17.1
Cooled for analysis	2.5	11.2	6.3	20.9
Increase due to sample preparation (%)	67	56	54	22

6.6.6 Fat as an artefact in the Prosky AOAC procedure

It is stated in the instructions for the Sigma Prosky AOAC Kit that dried samples with more than 10% fat should be defatted with petroleum ether before analysis. The fat contents of the dried samples of the ten food groups were obtained using a Foss-Let Oil Meter; only two samples, Meat products and Nuts, had fat contents in excess of 10% and these were defatted according to the Prosky AOAC procedure to obtain the values given in Table 1.

Fat may contribute to higher Prosky AOAC values even after following the recommended defatting procedure. To test that fat contents below 10% do not interfere, the Other vegetables sample was fat extracted and analysed following the Prosky AOAC procedure. This resulted in 7.4% loss of material The Prosky AOAC value for the extracted sample was 12.2(±0.5)g/100g DM, which represents a decrease of 21% from the value obtained for the non-defatted sample (Table 1). This raises the question of, to what extent the fat content in the other samples may also interfere. Further, Mongeau (1994) has demonstrated that the fat extraction technique used in the Prosky AOAC procedure is inadequate. He obtained a value of 30% 'DF' for a commercial pudding sample using the Prosky AOAC defatting procedure, but this was reduced to 5.6% 'DF' when chloroform/methanol was used for fat extraction.

6.6.7 Organic acids as an artefact in the Prosky AOAC procedure

Organic acids, such as tannins (tannic acid, ellagic acid, chlorogenic acid), citrate, ascorbate and phytate are not included in any definition of dietary fibre. However, due to their variable solubility in alcohol, some of these compounds represent a potential source of interference in the Prosky AOAC procedure. For example, oxalate, phytate and citrate are partially insoluble in 78% ethanol and therefore included in the Prosky AOAC residue.

To demonstrate the mechanism, samples (250 mg) of phytate, citrate and oxalate both with and without calcium were taken through the Prosky AOAC procedure. Table 11 shows clearly that organic acids are included in the Prosky AOAC measurement. The addition of calcium ions (molar ratio of Ca^{2+} to organic acid approx. 1:2) increased the amount of material that was included in the residue.

Table 11

Contribution of organic acids to Prosky AOAC values

Organic acid	No Ca^{2+}	With Ca^{2+}
Citrate	7.7 (0.3)	24.8 (0.5)
Oxalate	20.7 (1.9)	33.0 (7.0)
Phytate	22.1 (1.2)	31.5 (2.3)

The values are (g/100g DM).

6.6.8 Klason lignin as an artefact in the Prosky AOAC procedure

Together with many other minor non-carbohydrate constituents, lignin is an integral part of the plant cell wall. The extent of lignification may be important in estimating the digestibility of animal feed; however, no important physiological effect of lignin in the human diet has been demonstrated. Lignification of plant material may influence the solubility and fermentability of NSP but information on this will be obtained only if lignin is measured specifically and values are reported separately.

No accurate method is available for routine measurement of true lignin. What is often determined is a group of substances resistant to acid hydrolysis that are referred to as Klason lignin. The term is misleading because it encompasses lignin, other polyphenolic compounds, Maillard reaction products (MRPs) and degradation products formed as the result of heat processing. The Prosky AOAC method appears to include at least some Klason lignin in the residue (Table 12).

Table 12

The Klason lignin content of the Prosky AOAC residue

Food group	Klason lignin (g/100g DM)	
Bread	0.44	(0.44, 0.44)
Other cereals	0.32	(0.30, 0.34)
Meat products	1.56	(1.53, 1.59)
Green vegetables	1.69	(1.59, 1.79)
Potatoes	0.22	(0.02, 0.42)
Other vegetables	0.94	(0.94, 0.94)
Canned vegetables	0.68	(0.48, 0.88)
Fresh fruit	3.98	(3.88, 4.08)
Fruit products	1.41	(1.21, 1.61)
Nuts	0.63	(0.63, 0.63)

Average and individual values.

As part of the present study, values were obtained for Klason lignin for the ten food groups (Table 12). Not all Klason lignin is included in

Prosky AOAC values, because the procedure makes partial correction for MRPs by its correction for residual protein. The Klason lignin values in Table 12 are therefore likely to grossly overestimate the lignin included in the Prosky AOAC dietary fibre values. However, even Klason lignin values cannot account for the unidentified material in the Prosky AOAC residue for all the food groups (Table 4 and see Appendix II).

7.0 The Englyst NSP procedure

7.1 Stated aims

The stated aim of the Englyst NSP procedure is to measure plant cell-wall non-starch polysaccharides as the sum of their constituent sugars.

7.2 Principle

The Englyst procedure (Englyst *et al.*, 1994), which is an extension of the pioneering work of McCance & Widdowson and, later, Southgate, measures non-starch polysaccharides (NSP), using enzymic-chemical methods.

To obtain duplicate analysis of total NSP, two subsamples, defatted if containing more than 10% fat, are taken through the procedure. Following treatments in which starch is hydrolysed enzymically, and the NSP are precipitated with ethanol and hydrolysed with sulphuric acid, there are three options for the measurement of the constituent sugars. In the gas-liquid chromatography (GLC) option, the individual neutral sugars are measured by GLC and the uronic acids are measured by colorimetry. The high-pressure liquid chromatography (HPLC) option measures the individual neutral sugars and the uronic acids. In the colorimetry option, the neutral sugars and uronic acids are measured together.

The calculation for the GLC assay used here is:

NSP(g/100g DM) = (0.89 × Sum of neutral sugars) + (0.91 × Sum of uronic acids)

For the measurement of insoluble NSP, precipitation in ethanol is replaced by extraction for 30 minutes in aqueous buffer (pH 7). Soluble NSP = total NSP – insoluble NSP.

7.3 Results

The results of detailed analysis of the constituent sugars of the NSP of the ten food group samples are given in Table 13.

Table 13

Constituent sugars of the soluble, insoluble and total NSP content of the ten food groups.

		Constituent sugars (g/100g dry matter)								
		Rha	Fuc	Am	Xyl	Man	Gal	Glc	UAc	Total
Bread										
	Total	t	t	1.1	1.6	0.2	0.3	1.0	0.3	4.5
	Insoluble	t	t	0.5	0.8	0.1	t	0.7	0.1	2.2
	Soluble	t	t	0.6	0.8	t	0.3	0.3	0.2	2.3
Other cereals										
	Total	t	t	0.9	1.4	0.1	0.3	0.9	0.2	3.8
	Insoluble	t	t	0.4	0.7	0.1	0.1	0.5	0.1	1.9
	Soluble	t	t	0.5	0.7	t	0.2	0.4	0.1	1.9
Meat products										
	Total	t	t	0.2	0.2	0.1	0.2	0.4	0.1	1.1
	Insoluble	t	t	0.1	0.1	t	0.1	0.2	t	0.5
	Soluble	t	t	0.1	0.1	t	0.1	0.2	0.1	0.6
Green vegetables										
	Total	0.8	0.2	3.9	1.3	0.6	2.1	10.4	6.0	25.2
	Insoluble	0.2	0.1	1.2	1.0	0.5	0.9	10.3	0.7	14.9
	Soluble	0.6	t	2.7	0.2	t	1.2	0.1	5.3	10.3
Potatoes										
	Total	0.2	t	0.4	0.1	0.1	2.3	1.9	1.1	6.1
	Insoluble	t	t	0.1	0.1	0.1	0.4	1.9	0.1	2.7
	Soluble	0.2	t	0.3	t	t	1.9	t	1.1	3.4
Other vegetables										
	Total	0.3	t	1.0	0.5	0.5	1.2	3.7	2.8	10.0
	Insoluble	t	t	0.3	0.4	0.3	0.3	3.4	0.3	5.0
	Soluble	0.3	t	0.7	0.1	0.2	0.9	0.2	2.5	5.0
Canned vegetables										
	Total	0.2	0.1	2.7	1.3	0.3	0.8	4.1	1.8	11.3
	Insoluble	t	t	0.8	0.9	0.2	0.2	4.1	0.5	6.7
	Soluble	0.2	0.1	1.9	0.4	0.1	0.5	t	1.3	4.6
Fresh fruit										
	Total	0.2	0.1	1.0	0.7	0.3	0.7	2.7	3.0	8.7
	Insoluble	0.1	0.1	0.3	0.6	0.2	0.3	2.7	0.3	4.6
	Soluble	0.2	t	0.6	0.1	0.1	0.4	t	2.7	4.1
Fruit products										
	Total	0.1	t	0.3	0.3	0.1	0.3	0.9	1.0	3.1
	Insoluble	t	t	0.1	0.3	0.1	0.1	0.8	0.2	1.6
	Soluble	0.1	t	0.2	0.1	t	0.2	0.1	0.8	1.5
Nuts										
	Total	0.2	0.1	1.3	0.5	1.3	0.5	1.9	1.2	6.9
	Insoluble	0.1	t	1.0	0.4	1.3	0.3	1.7	0.7	5.5
	Soluble	0.1	t	0.3	0.1	t	0.2	0.2	0.5	1.4

t, trace. The values are the average of duplicate analyses.

The spectrum of the constituent sugars is characteristic for various types of plant NSP. Knowledge of the individual sugars may confirm the

identity of analytical samples and may indicate the origin of the NSP for unknown samples.

Detailed analysis (Englyst *et al.,* 1988,1989) has shown that in white flour products most of the fibre is present as soluble NSP, while in wholemeal products approximately two-thirds of the fibre is insoluble. The results in Table 13 indicate that wholemeal products do not represent a major component in either the Bread or Other cereal product groups. The soluble NSP glucose values for these groups are rather low, indicating that beta-glucans (found mainly in barley, rye and oats) are not present in large amounts. As expected, the values in Table 13 show that the NSP in the Bread and Other cereal groups contain more xylose than arabinose and only a small amount of uronic acids.

The presence of small amounts of arabinose and xylose in the Meat products group suggests that some plant products have been used in their formulation.

In contrast to cereal products, fruit and vegetables contain more arabinose than xylose and the majority of the arabinose is measured as soluble NSP. High values for uronic acids indicate a diet rich in fruit and vegetables.

For purposes where the detailed information on the constituent sugars is not required, values for total, soluble and insoluble dietary fibre may be obtained by the more rapid colorimetric end-point (Englyst *et al.,* 1994).

7.4 Are the stated aims achieved ?

For the majority of plant foods in the human diet, the method provides values for plant cell-wall NSP (Englyst & Cummings, 1984; Englyst *et al.,* 1982, 1987, 1992b, 1994). The starch, fat, organic acids, MRPs and Klason lignin that are included as artefacts in the Prosky AOAC procedure do not interfere with the specific measurement of the constituent sugars of NSP. Validation of the techniques used in the Englyst procedure has been described recently (Englyst *et al.,* 1992b, 1994; Quigley & Englyst, 1992, 1994).

7.5 Practical implications of the Prosky AOAC and Englyst methodologies

The Prosky AOAC procedure requires two crucibles with sintered glass filters for the analysis of each sample (i.e. 48 crucibles to measure 12 samples in duplicate) and a further four crucibles for the sample blank. Each sample is placed in a beaker and incubated with enzymes, followed by precipitation and washing of polymers with ethanol (in all, more than 17 litres of ethanol are used in the analysis of 12 samples compared with a little more than 3 litres in the Englyst procedure for the same number of samples). Material is transferred from the beakers to oven-dried, pre-weighed crucibles. Quantitative transfer can be extremely difficult to achieve, especially for samples that adhere to the walls of the beaker. Filtration times are very variable for different samples, and may be as long as six hours. During the procedure, seven separate weighings are required for each sample, so that a batch of 12 samples in duplicate and a reagent blank represents a total of 175 separate weighings, compared with the 24 required in the Englyst procedure. Even more weighings are required if samples have to be defatted.

Values are obtained by the Prosky AOAC procedure within a minimum of one and a half working days, or up to two and a half working days if filtration times are lengthy. The procedure is extremely labour-intensive, requiring manual transfer of material, many weighings, determination of ash content and Kjeldahl determination of total nitrogen. In contrast, with the Englyst NSP procedure, values for total, soluble and insoluble NSP may be obtained within one and a half working days using either of the chromatography end-points. Using the colorimetry end-point allows the determination of total, soluble and insoluble NSP within a working day. Agreement is good between the values obtained by the chromatographic and by the colorimetric assay, which is suitable for automation (Englyst & Hudson, 1987).

7.6 Related studies

7.6.1 Performance of the Prosky AOAC and Englyst procedures

The Prosky AOAC procedure and the Englyst procedure for the measurement of dietary fibre have been the subjects of several international collaborative trials. In the most recent trial (Wood *et al.*, 1993), 12 samples were analysed in duplicate; the mean total dietary fibre value for these samples obtained by the Prosky AOAC procedure was 21% higher than that obtained by the Englyst procedure, whilst the

mean value for soluble fibre measured by the Prosky AOAC method was only two-thirds of that measured by the Englyst procedure.

The precision of the methods is shown by values for repeatability (r-95) and reproducibility (R-95) for the variation within and between laboratories, respectively, where r-95 and R-95 are calculated as standard deviations × 2.8 (Table 14). Outliers by the Grubb's test at the $p<0.01$ level were excluded from the calculation of the mean and the precision data, but for all the methods less than 5% of the results were excluded. The precision data obtained with the Englyst procedure are superior to those obtained with the Prosky AOAC procedure.

Table 14

Repeatability and reproducibility of the Prosky AOAC and Englyst procedures in the MAFF trial

Method	Number of (labs)	Fibre content (mean)	Repeatability r-95 (mean)	Reproducibility R-95 (mean)
Englyst (GLC)				
Total	22	8.92	1.24	2.67
Soluble	22	3.83	1.36	2.01
Insoluble	22	5.09	0.95	1.92
Prosky AOAC (gravimetric)				
Total	16	10.82	2.11	5.34
Soluble	14	2.50	1.11	2.33
Insoluble	14	8.12	1.5	3.11

7.6.2 Dietary fibre solubility

The soluble fraction of fibre, which is fermented rapidly in the large intestine and contributes little to faecal bulking, has been implicated in lowering glycaemic response and cholesterol levels. Insoluble

components of fibre are fermented less readily and can have a marked effect on laxation.

The separation into soluble and insoluble fibre fractions is highly dependent on the pH used for extraction of the soluble portion. Table 15 shows values for total NSP and for the NSP that are soluble after extraction of a carrot preparation in aqueous buffer at pH 5 or at pH 7 for one hour at 100° C. All the uronic acids are extracted at pH 7, whereas only about half of the uronic acids are extracted at pH 5. The NSP glucose in carrot comes mainly from cellulose, which is highly insoluble and therefore not affected by the pH of the extraction medium. Overall, following extraction at pH 7, 63% of carrot NSP is measured as soluble dietary fibre. However, at pH 5 only 29% is soluble. In the Prosky AOAC procedure, the fraction that is soluble at pH 4.7 is extracted (*versus* pH 7 in the Englyst procedure), which may explain why the Prosky AOAC procedure gives lower values for soluble fibre. An extraction pH of 7 is used in the Englyst procedure because the pH of the small bowel is about 7 after a meal (Englyst & Cummings, 1990).

Table 15

Effect of pH on solubility of carrot NSP

	Total	Rha	Ara	Xyl	Man	Gal	Glc	UAc
Total NSP	73.7	2.6	6.2	1.1	1.3	9.2	21.3	32.0
Soluble at pH 5	21.3	0.5	0.8	0.1	0.1	1.6	0.8	17.4
Soluble at pH 7	46.2	2.2	4.5	0.1	0.1	6.7	0.7	31.9

NSP sugars were released by Saeman hydrolysis of unextracted material and material extracted at various pH values. All values are given as g/100gDM.

From Englyst (1985).

7.6.3 Resistant starch

Following a series of studies in man (Englyst & Cummings, 1985, 1986, 1987a) we have proposed a classification of starch for nutritional purposes (Englyst & Cummings 1987b; Englyst & Kingman, 1990; Englyst *et al.,* 1992a, 1996; Cummings *et al.,* 1995; and see Table 16). Resistant starch (RS) is defined as all the starch and starch degradation products that, on average, escape digestion and absorption in the human small intestine (Englyst & Cummings, 1990), and our measurement of RS is based on that definition (Englyst *et al.,* 1992a).

Table 16.

Nutritional classification of starch

Type of starch	Example of occurrence	Probable digestion in small intestine
Rapidly digestible starch (RDS)	Freshly cooked starchy food	Rapid, complete
Slowly digestible starch (SDS)	Most raw cereals	Slow but complete
Resistant starch (RS):		
RS_1 Physically inaccessible starch	Partly milled grain and seed	Resistant
RS_2 Resistant starch granules	Raw potato and banana	Resistant
RS_3 Retrograded starch	Cooled, cooked potato bread and corn flakes	Resistant

From Englyst & Cummings (1990).

Much of the retrograded starch formed as the as the result of food processing or sample preparation is included in the Prosky AOAC procedure. However, RS_1 (Table 16) is not included, because milling samples before analysis renders this fraction of starch accessible to the enzymes used in the Prosky AOAC procedure. Further, any RS_2 present in a sample will be gelatinised during the boiling step and thus made susceptible to enzymic hydrolysis and not included. The starch that is included in the Prosky AOAC residue is therefore not a measure of RS.

Table 17 shows the values obtained by the American Institute of Baking, New York, for the increase in Prosky AOAC 'DF' content of white flour by repeated autoclaving and cooling cycles (Ranhotra *et al.*, 1991). They showed that the values may be increased by four- to fivefold, "primarily due to the formation of resistant starch", but the heat treatment used in these studies will also result in the formation of Maillard reaction products and these will be included by the Prosky AOAC procedure (see above).

Table 17

Formation of "High-fiber White Flour" by repeated heating and cooling

	Prosky AOAC 'DF' (% DM)	
	Bread flours	Pastry flours
Untreated	2.7 (0.1)	2.4 (0.1)
Heated/cooked	11.2 (0.6)	12.4 (1.3)

Data from Ranhotra *et al.* (1991); the values are mean (SD) for five bread flours and five pastry flours.

7.6.4 Klason lignin

Theander & Westerlund (1993) have demonstrated the formation of Klason lignin by heat processing of wheat and potato (Table 18). The values are doubled by extrusion processing of wheat flour, and increased by up to sixfold by cooking potato. This increase is the result of the formation of Maillard reaction products and, as in the Prosky AOAC procedure, these are included in the Klason lignin fraction and hence in the fibre values obtained by the Uppsala procedure (Theander & Westerlund, 1993).

Table 18

The formation of Klason lignin by food processing

Sample	Klason lignin (%)
Wheat flour	
Untreated	0.2
Extruded at 168°C	0.4
Wheat wholemeal	
Untreated	1.4
Extruded at 180°C	2.8
Potato	
Raw	0.4
Boiled	1.2
Pressure cooked	2.6

8.0 Discussion

The human diet contains a range of chemically distinct carbohydrates and research as well as labelling for dietary carbohydrates, including dietary fibre, should be based on the classification and measurement of chemically identified components. Such values do not become obsolete and can be used in different combinations for different purposes. Table 19 shows the classification of food carbohydrates.

The purpose of food labelling for dietary fibre is to help the consumer in the choice of the unfortified, high-fibre diet recommended in nutritional guidelines. This diet is low in free sugars, salt and fat, and is a good source of a range of naturally occurring nutrients, including vitamins, minerals and anti-oxidants. All the properties of a high-fibre diet, including those related to the structural properties and the encapsulation of nutrients within plant cell walls, have been implicated in the protection from a variety of diseases (Trowell *et al.,* 1985; Southgate & Englyst, 1985; Spiller, 1993).

It is clear that any definition or measurement of dietary fibre must provide values that serve as a marker for the high-fibre diet embodied in the dietary fibre hypothesis if it is to be meaningful in terms of the evidence that exists in support of this hypothesis. The common characteristic of the plant foods that comprise a high-fibre diet is the presence of plant cell walls. This is what prompted Trowell (1972) to offer the following definition of dietary fibre material:

...the skeletal remains of plant cells that are resistant to hydrolysis by the enzymes of man.

This specific focus on the skeletal remains of plant cells deliberately excluded starch and other non-cell-wall material, and provided the source definition of dietary fibre as endogenous plant cell-wall material (Trowell, 1972; Trowell *et al.,* 1985). Any analytical procedure that was capable of measurements reflecting endogenous plant cell-wall material would thus provide values that were meaningful in terms of the dietary fibre hypothesis.

Non-starch polysaccharides (NSP) represent the principal component (approx. 90%; Selvendran & Robertson, 1990) of plant cell walls, and their measurement provides a good index of endogenous plant cell-wall material for most plant foods (Englyst *et al.,* 1987, 1994). NSP values thus provide a very good marker for the high-fibre diet embodied in the

dietary fibre hypothesis and recommended in the guidelines. The use of these values as Fibre for food labelling would aid the consumer in selecting the recommended foods.

The Prosky AOAC procedure is based on a definition of dietary fibre as the sum of indigestible polysaccharides and lignin. Within its stated aims, the residue obtained in the Prosky AOAC procedure can contain starch and NSP in any ratio, only NSP, only starch, or only lignin. However, the method does not measure inulin or all resistant starch, and appears to underestimate NSP (Table 4 and Appendix II). Therefore, the Prosky AOAC procedure does not achieve its stated aim to measure all indigestible polysaccharides. Further, we showed that for the food groups analysed here, up to 42% (see Table 4), and for individual foods up to 60% (Appendix II), of the analytical residue may be unaccounted for as polysaccharides and it is unreasonable to suggest that this can all be lignin.

NOVELOSE™ resistant starch (National Starch and Chemical Company) is being advertised as "A new tool for creating fiber-rich foods" (1995). NOVELOSE measures as approximately 30% 'DF' by the Prosky AOAC procedure but contains no plant cell-wall material and thus yields a value of zero for NSP. Apart from fermentation in the large intestine, resistant starch shares none of the properties traditionally associated with dietary fibre. The use by the food industry of material like NOVELOSE™ in the production of snack foods or breakfast cereals can result in apparently dramatic increases in "fibre" content if the values are obtained by the Prosky AOAC procedure.

Table 19

Classification of food carbohydrates

Type	Components	Hydrolysed and absorbed in the small intestine	Included in AOAC Englysts NSP[a]	Prosky residue[b]	Glycaemic response
Sugars	Glucose, fructose, sucrose, lactose	Mostly	No	No	Large
Sugar alcohols	Sorbitol, xylitol, lactitol, maltitol	Sparsely	No	No	0
Short-chain carbohydrates (SC) (soluble in 80% alcohol)	Resistant SC (RSC) (Fructo- and galacto-oligosaccharides pyrodextrins, polydextrose)	No	No	No	0
	Maltodextrins	Yes	No	No	Large
Starch	Rapidly digestible starch (RDS) [c]	Yes	No	No	Large
	Slowly digestible starch (SDS)	Yes	No	No	Small
	Resistant starch (RS)	No	No	Partly	0
Non-starch polysaccharides	Endogenous plant cell wall NSP[d] (Cellulose, hemicelluloses, pectins)	No	Yes	Yes	0*
	Other plant NSP (Gums, mucilages, isolated preparations)	No	Yes	Yes	0*

0* May affect the glycaemic response to other carbohydrates.
a Englyst *et al.* (1994).
b Official Methods of Analysis (1990).
c Includes maltodextrins (partly hydrolysed starch).
d Marker for the high-fibre diet embodied in the dietary fibre hypothesis.

Current intakes of RS are low (of the order of 3g per day in Europe) and the evidence for the beneficial effects of a high-fibre diet does not rely on measurements of RS. Studies are underway to determine the potential health benefits or hazards of RS but this is a separate issue from dietary fibre and specific measurement of RS is required to interpret such studies and to fully understand the role of RS in the diet (Englyst *et al.*, 1992a).

The Prosky AOAC values represent an unspecified mixture of NSP and starch (see Tables 3 and 4) and, like the Uppsala procedure (see Related Studies, Table 18), a range of substances, including MRPs, collectively and misleadingly termed Klason lignin (see Tables 6 to 9). Prosky AOAC

values can be manipulated by food processing techniques (Prosky & DeVries, 1992; see Table 5). These values are not interpretable in terms of chemistry, physiology or nutrition, and are divorced from the dietary fibre hypothesis and the evidence for the benefits to health of a high-fibre diet. The use of Prosky AOAC values, which can represent solely non-plant cell-wall material, for food labelling and in food tables can seriously mislead the consumer and those wishing to interpret intake data or formulate diets of specified fibre content.

The argument put forward by some of the food industry (Bär, 1994), that all the carbohydrates that reach the large intestine have "fibre-like" properties and should be included as dietary fibre, is not supportable. There is no justification for the inclusion of retrograded starch, as in the Prosky AOAC and Uppsala procedures, or indeed any indigestible carbohydrate other than the plant cell-wall NSP in the definition and measurement of dietary fibre. Many components of the diet, including protein and carbohydrate, reach the human large intestine, and all will have some effect upon faecal bulk. Carbohydrates other than plant NSP reach the large intestine (see Table 19); e.g. lactose in many adults, some sugar alcohols, fructo and galacto-oligosaccharides, resistant starch, and a range of semisynthetic compounds including pyrodextrins and polydextrose. However, of these substances only the plant cell-wall NSP are characteristic of the plant foods that constitute a high-fibre diet. The suggested inclusion of non plant cell-wall components in the definition and measurement of dietary fibre is not compatible with the evidence that exists for the health benefits of a high-fibre diet or the advice given in the dietary guidelines

With dietary fibre defined and measured for food labelling as endogenous plant cell-wall NSP, a dietary fibre intake of around 20 g/day from a mixture of fruit, vegetables and unfortified cereal products as recommended in the dietary guidelines should ensure a diet with a low energy density and rich in minerals, vitamins and anti-oxidants, and that has been shown to be protective against diabetes, coronary heart disease and some types of cancer. This high-fibre diet, rich in starch and low in fat, should be beneficial also in combating the alarming increase in the prevalence of obesity in Western countries.

9.0 Conclusions on dietary fibre

National dietary guidelines recommend an unfortified high-fibre diet rich in fruit, vegetables and wholemeal cereal products. A range of health

benefits has been shown for this diet, which is a good source of anti-oxidants, vitamins and minerals, and is low in fat and energy. The unique characteristic of the plant foods that constitute a high-fibre diet is the presence of naturally occurring cell-wall material.

The principal aim of food labelling for dietary fibre is to guide the consumer in selecting the type of high-fibre diet that is recommended in the guidelines. Some measure of plant cell-wall material is thus required.

The Prosky AOAC procedure is not specific for plant cell-wall material but includes substances that are formed by food processing and by treatment of analytical samples. Such values are not suitable for food labelling because they do not aid the consumer in choosing the recommended diet.

Values for dietary fibre based on the measurement of plant cell-wall NSP aid the consumer in choosing the type of high-fibre diet recommended in the dietary guidelines and are therefore appropriate for food labelling.

10.0 References

Bär, A. (1994). Defining dietary fibre for nutrition labelling purposes: a nutritional/physiological approach. In *Food Ingredients Europe: Conference Proceedings 1993.* Maarssen, The Netherlands: Expoconsult Publishers.

Cummings, J.H., Hudson, G.J., Quigley, M.E. & Englyst, H.N. (1995). Defining dietary fibre for nutrition labelling purposes: a nutritional/physiological approach. COST 92 Metabolic and physiological aspects of dietary fibre in food. Recent progress in the analysis of dietary fibre. Proceedings of a workshop held on 28 and 29 October, 1994 in Copenhagen. (Vedbaek), Denmark, pp 17-36 [A.Sørenson, K.E.Bach Knudsen, H.N. Englyst, E. Gudmand Høyer, and M. Nyman, editors, European Commission, Directorate-General XII Science, Research and Development], Luxembourg.

Englyst, H.N. (1985). Dietary polysaccharide breakdown in the gut of man. PhD thesis, University of Cambridge.

Englyst, H.N. & Cummings, J.H. (1984). Simplified method for the measurement of total non-starch polysaccharides by gas-liquid chromatography of constituent sugars as alditol acetates. *Analyst* **109**, 937-942.

Englyst, H.N. & Cummings, J.H. (1985). Digestion of the polysaccharides of some cereal foods in the human small intestine. *American Journal of Clinical Nutrition* **42**, 778-787.

Englyst, H.N. & Cummings, J.H. (1986). Digestion of the carbohydrates of banana *(Musa paradisiaca·sapientum)* in the human small intestine. *American Journal of Clinical Nutrition* **44**, 42-50.

Englyst, H.N. & Cummings, J.H. (1987a). Digestion of polysaccharides of potato in the small intestine of man. *American Journal of Clinical Nutrition* **45**, 423-431.

Englyst, H.N. & Cummings, J.H. (1987b). Resistant starch, a 'new' food component: a classification of starch for nutritional purposes. In: *Cereals in a European Context. 1st*

European Congress of Food Science and Technology. pp. 221-233 [I.D. Morton, editor]. Chichester, England: Ellis Horwood.

Englyst, H.N. & Cummings, J.H. (1990). Dietary fibre and starch: definition, classification and measurement. In *Dietary Fibre Perspectives: Reviews and Bibliography* pp. 3-26 [A.R. Leeds, editor]. London: John Libbey.

Englyst, H.N. & Hudson, G.J. (1987). Colorimetric method for routine measurement of dietary fibre as non-starch polysaccharides. A comparison with gas-liquid chromatography. *Food Chemistry,* **24**, 63-76.

Englyst, H.N. & Kingman, S.M. (1990). Dietary fibre and resistant starch. A nutritional classification of plant polysaccharides. In *Dietary Fibre.* pp. 49-65 [D. Kritchevsky, C. Bonfield and J.W. Anderson, editors]. New York: Plenum Press.

Englyst, H.N., Cummings, J.H. & Wood, R. (1987). Determination of dietary fibre in cereals and cereal products-collaborative trials. Part III: study of further simplified procedures. *Journal of the Association of Public Analysts* **25**, 73-110.

Englyst, H.N., Bingham, S.A., Runswick, S.A., Collinson, E. & Cummings, J.H. (1989). Dietary fibre (non-starch polysaccharides) in fruit, vegetables and nuts. *Journal of Human Nutrition and Dietetics* **1**,247-286.

Englyst, H.N., Bingham, S.A., Runswick, S.A., Collinson, E. & Cummings, J.H. (1990). Dietary fibre (non-starch polysaccharides) in cereal products. *Journal of Human Nutrition and Dietetics* **2**, 253-271.

Englyst, H.N Kingman, S.M. & Cummings, J.H. (1992a). Classification and measurement of nutritionally important starch fractions. *European Journal of Clinical Nutrition* ***46,*** S33-S50.

Englyst, H.N., Quigley, M.E., Hudson, G.J. & Cummings, J.H. (1992b). Determination of dietary fibre as non-starch polysaccharides with gas-liquid chromatography. *Analyst* **117**, 1707-1714.

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

Englyst, H.N., Veenstra, J. & Hudson, G.J. (1996). Measurement of rapidly available glucose (RAG) in plant foods: a potential in vitro predictor of the glycaemic response. *British Journal of Nutrition,* **75**, 327-337.

Maillard, L.C. (1912). Action des acides amines sur le sucres; formation des melanoidines par voie Methodique. *C.R. Hebd. Seances Acad. Sci.* **154**, 66-68.

Mongeau, R. (1994). In *Transcript of the FDA Initiated Conference on Dietary Fiber and Cancer and Heart Disease,* 12-13 May 1994, Arlington, VA.

NOVELOSE (1995). Advertisement on the back cover of *Cereal Foods World* **40(5)**.

Official Methods of Analysis (1990). 15th Edit., vol. 11, section 985.29, p. 1105, Prosky AOAC, Arlington VA.

Prosky, L. & DeVries, J. (1992). *Controlling Dietary Fiber in Food Products.* New York: Van Nostrand Reinhold.

Quigley, M.E. & Englyst, H.N. (1992). Determination of neutral sugars and hexosamines by high-performance liquid chromatography with pulsed amperometric detection. *Analyst* **117**, 1715-1718.

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

Ranhotra, G.S., Gelroth, J.A. & Eisenbraun, G.J. (1991). High-fiber white flour and its use in cookie products. *Cereal Chemistry* **68**, 432-433

Selvendran, R.R. & Robertson, J.A. (1990). The chemistry of dietary fibre: an holistic view of the cell wall matrix. In *Dietary Fibre: Chemical and Biological Aspects. Royal Society of Chemistry Special Publication no. 83,* pp. 27-43 [D.A.T. Southgate, K. Waldron, I.T. Johnson and G.R. Fenwick, editors]. Cambridge, UK: Royal Society of Chemistry.

Southgate, D.A.T. & Englyst, H.N. (1985). Dietary fibre: chemistry, physical properties and analysis. In *Dietary Fibre, Fibre-depleted Foods and Disease,* pp. 31-55 [H. Trowell, D. Burkitt and K. Heaton, editors]. London: Academic Press.

Spiller G.A. (1993). CRC Handbook of Dietary Fiber in Human Nutrition, 2nd edition. Boca Raton, FA: CRC Press.

Theander, O. & Westerlund, E. (1993). Determination of individual components of dietary fiber. In *CRC Handbook of Dietary Fiber in Human Nutrition,* pp. 77-98 [G.A. Spiller, editor]. Boca Raton, FA: CRC Press Inc.

Trowell, H. (1972). Ischaemic heart disease and dietary fibre. *American Journal of Clinical Nutrition* **25,** 926-932.

Trowell, H., Burkitt, D. & Heaton, K.W. (1985). Editors of *Dietary Fibre, Fibre-depleted Foods and Disease.* London: Academic Press.

Weisburger, J.H. (1994). Specific Maillard reactions yield powerful mutagens and carcinogens. In *Maillard Reactions in Chemistry, Food, and Health.* pp. 335-340 [T.P. Labuza, G.A. Reinecius, V.M. Monnier, J. O'Brien and J.W. Baynes, editors]. Cambridge, UK: Royal Society of Chemistry.

Wood, R., Englyst, H.N., Southgate, D.A.T. & Cummings, J.H. (1993). Determination of dietary fibre in foods-collaborative trials. IV. Comparison of Englyst GLC and colorimetric measurement with the Prosky procedure. *Journal of the Association of Public Analysts* **29**, 57-141.

APPENDIX I

Ash and 'protein' (N × 6.25) correction of the Prosky AOAC residues for the ten food groups. The values in parentheses are SD (n = 4) for the residue weights and ± around the average of two analyses for Ash, N × 6.25 and Prosky AOAC 'DF' values.

Sample	Fat corr*	Residue		Ash		N × 6.25		Prosky AOAC 'DF'	
		(mg/g DM)						(g/100g DM)	
Bread	-	81.8	(2.1)	10.2	(1.5)	12.6	(1.4)	5.9	(0.2)
Other cereals	-	70.1	(5.3)	8.0	(2.3)	11.7	(2.4)	4.8	(0.1)
Meat products	0.656	189.6	(4.9)	20.4	(0.3)	155.4	(6.1)	1.1	(<0.1)
Green vegetables	-	389.3	(19.3)	32.4	(<0.1)	49.7	(12.3)	30.2	(0.4)
Potatoes	-	99.9	(7.1)	12.1	(5.8)	8.9	(1.3)	7.6	(0.2)
Other vegetables	-	217.2	(15.7)	17.9	(1.9)	14.7	(0.5)	17.7	(0.1)
Other vegetables	0.926	160.7	(8.2)	13.0	(1.7)	15.5	(0.7)	12.1	(0.5)
Canned vegetables	-	228.4	(5.7)	18.6	(6.1)	35.4	(1.4)	16.9	(0.6)
Fresh fruit	-	140.5	(3.6)	5.6	(1.3)	11.6	(1.4)	12.1	(0.2)
Fruit products	-	54.8	(2.7)	8.0	(3.1)	5.2	(0.8)	4.0	(0.3)
Nuts	0.528	339.8	(15.9)	38.7	(4.0)	133.5	(4.6)	9.2	(0.3)

*Correction factor for the decrease in weight of the freeze-dried sample upon defatting.

The Meat products and Nuts samples were defatted before analysis according to the Prosky AOAC procedure. The Other vegetables sample was analysed both before and after defatting. Although the fat removed from the freeze-dried material (7.4%) was below the level at which the Prosky AOAC procedure recommends defatting (10%), there is a considerable difference in the Prosky AOAC value obtained for this food group before defatting, 17.7%, and that obtained after defatting, 12.1%.

For non-defatted samples, Prosky AOAC 'DF' is calculated as:

$$100 \times \{ [\text{Residue weight} - (\text{Ash} + \text{Protein} + \text{Blank})]/\text{Sample weight}\}$$

For defatted samples, Prosky AOAC 'DF' is calculated as:

$$100 \times \{ [\text{Residue weight} - (\text{Ash} + \text{Protein} + \text{Blank})] \times \text{Fat corr}/\text{Sample weight}\}$$

APPENDIX II

Polysaccharides and unidentified material in the Prosky AOAC residue for the 12 MAFF trial samples

Sample	Englyst NSP	NSP in Prosky AOAC residue	Prosky AOAC 'DF' (g/100g DM)	Total polysaccharides in the residue (g/100g DM)	Unidentified material (g/100g DM)	(% 'DF')
Apple	12.3	11.5	12.7	11.6	1.1	9
Baked beans	12.7	12.1	16.8	14.2	2.6	15
Banana	4.3	3.3	9.0	3.6	5.4	60
Bread mix	6.3	6.1	9.0	7.0	2.0	22
Cabbage	26.7	23.8	27.3	23.8	3.5	13
Coconut	11.5	10.9	16.0	10.9	5.1	32
Corn flakes	1.2	0.6	3.1	2.5	0.8	26
Porridge	6.8	6.4	6.7	6.6	0.1	1
Potato	6.8	6.4	7.6	6.9	0.7	9
Rye bread	5.4	5.1	6.7	5.9	0.8	12
White bread	3.1	2.9	4.7	3.8	0.9	19
Wholemeal bread	9.4	9.0	11.4	9.9	1.5	13

Postscript on Resistant Starch and Dietary Fibre

The arrival in the marketplace of resistant starch (RS) products such as Novelose™ illustrates the ability to use the Prosky AOAC Prosky procedure to introduce huge amounts of RS into the human diet under the guise of dietary fibre. However, there is no evidence that RS confers any of the health benefits associated with a naturally high-fibre diet.

The consequences to health of long-term, high-level intakes of RS are unknown. Some experimental studies have identified potential benefits associated with the consumption of RS. For example, fermentation of RS in the human large intestine has been shown to reduce faecal ammonia (Birkett *et al.,* 1996) and the short-chain fatty acids produced by fermentation of carbohydrates, including RS, have been implicated as a protective factor against colon cancer (e.g. van Munster *et al.,* 1994). Other studies, however, have shown that RS can enhance tumour formation in rats (Young *et al.,* 1996), and Burn *et al.* (1996) have shown the highly significant enhancement of cancer formation by RS in a mouse model.

Current European diets provide an average of about 3 g of RS per capita per day and there is no evidence that this amount is detrimental to health. Neither is there any evidence that high levels of RS intake are beneficial to human health, and the two animal studies (Young *et al.,* 1996; Burn *et al.,* 1996) indicate that RS in elevated amounts may be detrimental to health.

Although detrimental effects have been shown only in animal studies, increasing the amount of RS in foods above current levels should be avoided pending the outcome of further studies of possible benefits or hazards.

Dietary fibre may affect the rate and extent of starch digestion in the small intestine but, as pointed out by Trowell (Trowell, 1972; Trowell *et al.,* 1985) and by Johnson & Southgate (1993), starch is totally divorced from the dietary fibre concept. In line with this, the EC Scientific Committee for Food stated in a report of their deliberations on the definition of dietary fibre for nutrition labelling,(Commission of the European Communities, 1993):

> *"There was lengthy discussion as to the material that should be defined as fibre for the purposes of nutrition labelling. In particular the inclusion or not of Resistant Starch in the definition of fibre was argued extensively. The Committee decided that the material to be considered as fibre for the purposes of nutrition labelling should be confined to non-starch polysaccharides of cell-wall origin."*

The national dietary guidelines provide nutritional advice based on proven beneficial relationships between diet and health. It is important that the consumer can understand the information on nutrition labels in terms of this

advice. The guidelines recommend that an adequate intake of dietary fibre should be obtained by the consumption of a range of fruits, vegetables and whole-grain cereal products. This naturally high-fibre diet is known to be associated with a low incidence of Western diseases and the guidelines discourage the use of isolated fibre preparations ("fibre supplements") as a source of dietary fibre. Food labelling for Fibre must be compatible with the dietary advice and therefore must be a measure of naturally occurring fibre in plant foods if the consumer is to be aided in the choice of foods recommended in the guidelines.

If resistant starch and other non-plant-cell-wall materials were to be included, Fibre values would be highly dependent on food processing. The consumer would be misled into selecting highly processed foods, with artificially elevated "Fibre" values. Such values (e.g. those obtained by the Prosky AOAC Prosky procedure) do not provide reliable guidance for the consumer in the selection of a naturally high-fibre diet. However, Fibre labelling that is based on the measurement of plant cell-wall NSP is in line with the nutritional advice, provides reliable guidance for the consumer and makes an important contribution to the promotion of public health.

References

Birkett, A., Muir, J., Phillips, J., Jones, G. & O'Dea, K. (1996). Resistant starch lowers faecal concentrations of ammonia and phenols in humans. *American Journal of Clinical Nutrition,* **63**, 766-772.

Burn, J., Katheuser, A., Fodde, R., Coaker, J., Chapman, P.D. & Mathers, J.C. (1996). Intestinal tumours in the Apc mouse: aspirin not protective and resistant starch increases small bowel tumours. *European Journal of Human Nutrition,* **4 (suppl. 1)**, 13.

Commission of the European Communities, Directorate Industry, III/E/1. Scientific Committee for Food, Document III/5481/93-EN-FINAL, Brussels, 21 October 1993.

Johnson, I.T. & Southgate, D.A.T. (1993). *Dietary Fibre and Related Substances, London,* Chapman and Hall.

Trowell, H. (1972). Ischaemic heart disease and dietary fiber. *American Journal of Clinical Nutrition,* 25, 926-932.

Trowell, H. Burkitt D. & Heaton, K. (1985). Editors of *Dietary Fibre, Fibre-depleted Foods and Disease,* London: Academic Press.

van Munster, I.P., Tangerman, A. & Nagengast, F.M. (1994). Effect of resistant starch on colonic fermentation, bile-acid metabolism, and mucosal proliferation. *Digestive Diseases and Sciences,* **39**, 834-842.

Young, G.P., Mclntyre, A., Albert, V., Folino, M., Muir, J.G. & Gibson, P.R. (1996). Wheat bran suppresses potato starch-potentiated colorectal tumorigenesis at the aberrant crypt stage in a rat model. *Gastroenterology,* **110**, 508-514.

J.Assoc.Publ.Analysts, 1996, 32,39-52

MAFF VALIDATED METHOD

V37

METHOD FOR THE DETECTION OF IRRADIATED FOOD CONTAINING FAT

The Department of Agriculture for Northern Ireland and the Queen's University of Belfast Chemical Method for Detection of 2-Dodecylcyclobutanone and 2-Tetradecylcyclobutanone in Irradiated Food Containing Fat

Correspondence on this method may be sent to Eileen Stewart, Food Science Division, The Department of Agriculture for Northern Ireland and the Queen's University of Belfast, Newforge Lane, Belfast, BT9 5PX.

Correspondence on the MAFF Validated Methods Series may be sent to Roger Wood, Food Labelling and Standards Division, Ministry of Agriculture, Fisheries and Food, CSL Food Science Laboratory, Norwich Research Park, Colney, Norwich, NR4 7RG

COSHH AND SAFETY CONSIDERATIONS

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 Codes of Practice, Control of Substances Hazardous to Health Regulations, 1988") must be made before using this method.

Each laboratory should follow its own safety rules and national regulations, particularly COSHH and IRRs, with respect to the sample preparation and measurement.

1. SCOPE AND FIELD OF APPLICATION

This method permits the identification of irradiated food containing fat. It has been successfully tested in interlaboratory trials on raw chicken, pork, and liquid whole egg.

2. DEFINITION

Irradiated foods containing fat comprise those which have been exposed to gamma radiation or machine sources of ionising radiation (e.g. linear accelerator). Positive results imply that a sample has been so irradiated at a dose exceeding the minimum detectable dose. The method described

is based on the mass spectrometric detection of radiation-induced 2-alkylcyclobutanones after gas chromatographic separation.

3. PRINCIPLE

During irradiation, the acyl-oxygen bond in triglycerides is cleaved and this reaction results in the formation of 2-alkylcyclobutanones containing the same number of carbon atoms as the parent fatty acid and the alkyl group is located in ring position 2. Thus, if the fatty acid composition is known, the 2-alkylcyclobutanones formed can be predicted.

The 2-alkylcyclobutanones which were analysed in interlaboratory studies were 2-dodecylcyclobutanone (DCB) and 2-tetradecyl-cyclobutanone (TCB) which are formed from palmitic and stearic acid respectively, during irradiation. To date, there is no evidence that the 2-alkylcyclobutanones can be detected in unirradiated food. The 2-alkylcyclobutanones are extracted using n-hexane along with the fat. The extract is then fractionated using adsorption chromatography prior to separation using gas chromatography (GC) and detection with a mass spectrometer (MS).

4. REAGENTS

During analysis use only reagents of recognized analytical grade, the purity of which has to be tested regularly by analysis of blank samples.

4.1 n-Hexane.

4.2 Sodium sulfate, anhydrous.

4.3 Diethyl ether.

4.4 Stock standard solutions, n-hexane or isooctane may be used to prepare solutions of 2-cyclohexylcyclohexanone (5 μg/ml), and 2-dodecyl-cyclobutanone and 2-tetradecylcyclobutanone (100 μg/ml).

Store at -20°C.

4.5 Working standard solutions, n-hexane is used to prepare solutions of 2-cyclohexylcyclohexanone (0.5 μg/ml) (internal standard), 2-dodecyl-cyclobutanone and 2-tetradecylcyclobutanone (10 μg/ml).

Store at -20°C.

4.6 **Water, double glass distilled.**

4.7 **Florisil**, 150 μm to 250 μm (60 mesh to 100 mesh), pesticide residue analysis grade. Before use, activate the adsorbent by heating at 550°C for at least 5 h or overnight. Cool in a desiccator. Keep well sealed after cooling. Prepare deactivated Florisil by adding 20 parts of water (**4.6**) to 100 parts of adsorbent (w/w). Approximately 30 g of activated Florisil is required to prepare sufficient deactivated absorbent for each column. Ensure that the deactivated Florisil contains no lumps and that the powder flows freely. Leave to equilibrate overnight. Use within one week.

4.8 **Nitrogen**, for concentrating solutions.

4.9 **Helium**, as carrier gas.

5. APPARATUS

Usual laboratory apparatus and, in particular, the following:

5.1 **Electric blender.**

5.2 **Soxhlet apparatus**, with suitable flask of 250 ml and extractor of 100 ml.

5.3 **Cellulose extraction thimbles**, e.g. of length 80 mm to 100 mm, with an internal diameter of 30 mm.

5.4 **Cotton wool**, non-absorbent.

5.5 **Electric heating mantle** or **water-bath.**

5.6 **Chromatography column**, having a length of 300 mm and with an internal diameter of 20 mm, fitted with a frit, a polytetrafluoroethylene (PTFE) stopcock and a ground glass joint at the top.

5.7 **Separating funnel**, e.g. of 250 ml, with a ground glass joint.

5.8 **Rotary evaporator.**

5.9 **Apparatus for concentration of solutions** under nitrogen.

5.10 **Gas chromatograph (GC) glass vials.**

5.11 **Capillary column**, with suitable performance characteristics, see Annex I.

5.12 **Gas chromatograph (GC) linked to a mass spectrometer (MS).**

6. PROCEDURE

6.1 Sampling technique

When taking samples, give preference to those parts of the food which have a high fat content, e.g. chicken skin. Keep the sample in a sealable glass vessel or in fat-free metal foil.

6.2 Sample preparation

Coarsely chop the samples of food and then homogenise in an electric blender (**5.1**). For liquid whole egg, ensure that the sample is thoroughly mixed prior to sampling.

6.3 Fat extraction

Weigh 20 g of anhydrous sodium sulfate (**4.2**) and 20 g of well mixed homogenised sample into an extraction thimble (**5.3**), mix and plug with cotton wool (**5.4**). Extra sodium sulfate may be used if necessary. It is recommended that liquid egg is dried at 100 °C for 12 h prior to extraction. A thin film of egg partially dried (2 h at 100°C) has given comparable results. Alternative drying procedures, e.g. freeze-drying, may be used provided recovery of 2-alkylcyclobutanones is checked (see **6.7**).

Pour 100 ml of n-hexane (**4.1**) into a 250 ml flask (**5.2**) and place extractor on top. Place extraction thimble in the extractor and add 40 ml of n-hexane. Place the flask on the heating mantle (**5.5**) and condenser on top of the extractor. Reflux and extract gently for 6 h. The solvent should siphon over four times in approximately 1 h. Remove the flask from the heat and dispose of the thimble and the n-hexane in the extractor. Transfer the solvent from the flask to a 100 ml stoppered cylinder and adjust the volume to 100 ml with more solvent. Add 5 g to 10 g of anhydrous sodium sulfate, stopper, mix and leave overnight.

6.4 Determination of lipid content

Dry duplicate flasks for at least 4 h or overnight at 100°C. Cool and weigh. Pipette an aliquot of lipid extract into each flask, rotary evaporate to dryness. Dry for at least 4 h or overnight at 100°C and reweigh. Alternatively, to provide a more rapid measurement of lipid content, pipette an aliquot of lipid extract into a glass vial, the weight of which has been determined. Evaporate the solvent under a stream of nitrogen. Reweigh. Repeat the process until the weight is constant. Calculate the volume of extract required to provide approximately 200 mg of lipid. Record the exact weight of lipid applied to the column.

6.5 Florisil column chromatography

Prepare a Florisil column (200 mm to 210 mm) using a chromatography column (**5.6**), deactivated Florisil (**4.7**) and n-hexane (**4.1**). Allow the n-hexane level to drop to just above the top of the Florisil.

Take a volume of the extract which provides approximately 200 mg of lipid and concentrate if necessary. The final volume should not exceed 5 ml.

Apply the lipid extract, rinse the flask with approximately 5 ml of n-hexane and apply to the column. Allow the n-hexane level to drop to just above the top of the Florisil and add 5 ml to 10 ml of n-hexane. Place the remaining n-hexane (150 ml in total) in the separating funnel (**5.7**) on top of the column, elute at 2 ml/min to 5 ml/min and collect the eluent in a suitable 250 ml flask.

When the funnel is empty (take care that the column does not run dry), change the collection flasks and elute with 150 ml of 1% diethyl ether (**4.3**) in n-hexane. Rotary evaporate the 1% diethyl ether fraction at 40°C, using minimum vacuum, to 5 ml to 10 ml and transfer to a test tube. Concentrate to dryness under a stream of nitrogen at 40°C ensuring that the sample is not left under nitrogen flow once it is dry. Resuspend immediately in 200 μl of a solution of 2-cyclohexylcyclohexanone (**4.5**).

6.6 Separation and detection.

Separate the 2-alkylcyclobutanones using a suitable capillary column (**5.11**) and identify using a mass spectrometer (**5.12**) operating in the selected ion monitoring mode for ions m/z 98 and m/z 112. In A.1 (Appendix I) an example of the GC/MS conditions used with an Ultra 1 column is given. Figures A.2 and A.3 (Appendix I) show the typical electron impact mass spectra of DCB and TCB. Figures A.4 to A.6 (Appendix I) show typical chromatograms for irradiated chicken, pork and egg.

6.7 Internal quality control

Test the system using an unirradiated control sample of the same type as the unknown sample and a duplicate control spiked with 200 μl and 100 μl of 10 μg/ml DCB and TCB in n-hexane or isooctane respectively. Spiking should be done immediately after sample preparation (**6.2**) and prior to further treatment. Treat these samples in the same way as the

unknown samples. Calculate the percentage recovery using the spiked sample.

Check the linear range regularly using standard solutions of suitable concentrations (e.g. 0.25 μg/ml up to 2 μg/ml of DCB or TCB with 0.5 μg/ml 2-cyclohexylcyclohexanone (**4.5**)).

7. EVALUATION

7.1 Identification of 2-alkylcyclobutanones

DCB produces peaks of ions m/z 98 and m/z 112 in a ratio of approximately 4.0 - 4.5 to 1 while for TCB the corresponding ratio is approximately 3.8 - 4.2 to 1. The ratios in samples should reflect those found in standards analysed at the same time. Both ions m/z 98 and m/z 112 have to be present in the correct ratio to give a positive identification. The signal to noise ratio of both ions monitored should be greater than 3 to 1 and the relative ion intensities should be within 20% of those obtained from injection of a standard of similar concentration run on the same day. In the case of positive results, scan, e.g. between ions m/z 95 and m/z 115, to confirm that ions m/z 98 and m/z 112 are the major ions present at the retention times of the standard 2-alkylcyclobutanones.

7.2 Calculation of the content of 2-alkylcyclobutanones

Measure a number of standard solutions (e.g. 3) containing DCB and TCB (**6.7**) either side of the samples.

Calculate the relative response F of each 2-alkylcyclobutanone in relation to the internal standard (**4.5**) by equation (1).

$$F = \frac{A_{cy}}{A_{is} \times \rho_{cy}} \qquad (1)$$

where:

A_{cy} *is the peak area of ion m/z 98 of 2-alkylcyclobutanone;*

A_{is} *is the peak area of ion m/z 98 of the internal standard (see 6.7);*

ρ_{cy} *is the mass concentration of 2-alkylcyclobutanone, in micrograms per millilitre.*

Average all the responses F to get F_{av} for each of the 2-alkylcyclobutanones. Calculate the mass concentration, $P_{cy/s}$ in micrograms per 200 microlitres of both 2-alkylcyclobutanones, respectively, as follows:

$$\rho_{cy/s} = \frac{A_{cy/s}}{A_{is/s} \times F_{av} \times 5} \qquad (2)$$

where:

$A_{cy/s}$ *is the peak area of ion m/z 98 corresponding to 2-alkylcyclobutanone in the sample;*

$A_{is/s}$ *is the peak area of ion m/z 98 corresponding to the internal standard in the sample;*

F_{av} *is the average of all ratios F as calculated using equation (1).*

Correction for lipid as follows:

$$W_{cy} = \frac{\rho_{cy/s}}{m_o} \times 1000 \qquad (3)$$

where:

$\rho_{cy/s}$ *is the mass concentration of 2-alkylcyclobutanone in the sample in micrograms per 200 microlitres, as calculated using equation (2);*

m_o *is the weight of the lipid taken, in milligrams;*

w_{cy} *is the mass fraction of the corresponding 2-alkylcyclobutanone, in micrograms per gram lipid.*

7.3 Identification of irradiated samples

Samples are considered to be irradiated when:

a) at least one 2-alkylcyclobutanone has been positively identified (**7.1**) and

b) the estimated concentration (**7.2**) exceeds the concentration equivalent to a signal to noise ratio of 3 to 1 in the least sensitive ion.

8. VALIDATION

The procedure as described in this protocol has been subjected to international collaborative trials organised by the Department of Agriculture for Northern Ireland and the Queen's University of Belfast under the auspices of the Community Bureau of Reference (BCR) and the Food and Agriculture Organisation (FAO)/International Atomic Energy Agency (IAEA). A summary of the results obtained is given in Appendix II, Tables 1 and 2. The results indicated that clear differentiation is obtained between the unirradiated and irradiated samples used in the trials. Detection of irradiated raw chicken has been validated for doses of approximately 0.5 kGy and above. The detection of irradiated liquid whole egg and raw pork has been validated for doses of approximately 1.0 kGy and above. Validation at these doses covers the majority of commercial applications.

9. REFERENCES

9.1 Stevenson, M.H. 1992. Progress in the identification of irradiated food, Trends in Food Sci. and Technol., **3**, 257-262.

9.2 Crone, A.V.J., Hand, M.V., Hamilton, J.T.G., Sharma, N.D., Boyd, D.R. and Stevenson, M.H. 1993. Synthesis, characterisation and use of 2-tetradecylcyclobutanone together with other cyclobutanones as markers for irradiated liquid whole egg, J. Sci. Food Agric., **62**, 361-367.

9.3 Stevenson, M.H. 1994. Detection of irradiated food, Food Technol., **48 (5)**, 141-144.

9.4 Meier, W. and Stevenson, M.H. 1993. Determination of volatiles and o-tyrosine in irradiated chicken. Results of an intercomparison study. In "Recent Advances on the Detection of Irradiated Food", Eds. M. Leonardi, J. J. Raffi and J. J. Belliardo. BCR Information, Luxembourg, Commission of the European Communities, Report EUR 14315 EN, p. 211-218,

9.5 Stevenson, M.H., Meier, W., and Kilpatrick, D.J. 1994. A European Collaborative Blind Trial Using Volatile Hydrocarbons and 2-Dodecylcyclobutanone to Detect Irradiated Chicken Meat. BCR Information, Luxembourg, Commission of the European Communities, Report EUR 15969 EN.

9.6 Stevenson, M.H., Kilpatrick, D.J. and Mc Murray, C.H. 1994. FA0/IAEA Collaborative Blind Trial Using 2-Dodecylcyclobutanone and 2-Tetradecylcyclobutanone to Detect Irradiated Chicken, Pork and Liquid Whole Egg, FA0/IAEA Report, Vienna, Austria.

9.7 Stevenson, M.H., Crone, A.V.J. and Hamilton, J.T.G. 1990. Irradiation detection, Nature, **344**, 202-203.

9.8 Boyd, D.R., Crone, A.V.J., Hamilton, J.T.G., Hand, M.V., Stevenson, M.H. and Stevenson, P.J. 1991. Synthesis, characterisation and possible use of

2-dodecylcyclobutanone as a marker for irradiated chicken. J. Agric. Food Chem., **39**, 789-792.

9.9 Crone, A.V.J., Hamilton, J.T.G. and Stevenson, M.H. 1992. Effect of storage and cooking on the dose response of 2-dodecylcyclobutanone, a potential marker for irradiated chicken, J. Sci. Food Agric., **58**, 249-252.

9.10 Crone, A.V.J., Hamilton, J.T.G. and Stevenson, M.H. 1992. The detection of 2-dodecylcyclobutanone in radiation-sterilised chicken stored for several years. Int. J. Food Sci. Technol., **27**, 691-696.

9.11 Raffi, J., Delincée, H., Marchioni, E., Hasselmann, C., Sjoberg, A.-M., Leonardi, M., Kent, N., Bögl, K.W., Schreiber, G.A., Stevenson, H. and Meier, W. 1994. Concerted Action of the Community Bureau of Reference on Methods of Identification on Irradiated Foods. BCR-lnformation, Luxembourg, Commission of the European Communities, Report EUR 15261 EN.

10. ACKNOWLEDGMENTS

The method described here was developed at the Food Science Division of the Department of Agriculture for Northern Ireland and the Queen's University of Belfast with support from MAFF under the direction of Dr. M. Hilary Stevenson. The method has been validated internationally in collaborative trials performed within a project of BCR, a research programme of the Commission of the European Communities, and also within the FAO/IAEA coordinated programme on Analytical Detection Methods for the Irradiation Treatment of Foods (ADMIT).

APPENDIX I

In the examples shown in A.4 to A.6, the 2-alkylcyclobutanones were separated using a Hewlett-Packard Ultra 1 column, 12 m ± 0.20 mm (i.d) with a 0.33 μm stationary phase (100 % dimethyl polysiloxane) in a Hewlett-Packard 5890 gas chromatograph directly linked to a Hewlett-Packard 5970B mass selective detector (MSD).

A.1 An example of the GC/MS conditions used with an Ultra 1 column

Temperature programme:	
Injector	250 °C
Transfer line	280 °C
Oven temperature programme	55 °C for 1 min.
	15 °C/min. to 300 °C
	300 °C for 5 min.
Injection volume	1 μl
Injection mode	splitless
Solvent delay	6 min.
Multiplier voltage	autotune value
Carrier gas He,	1 ml/min.
MSD selected ion monitoring of ions m/z 98 and m/z 112	
Electron impact ionisation	70 eV
Dwell time	50 ms

Figure A. 2

Electron impact mass spectrum of DCB standard

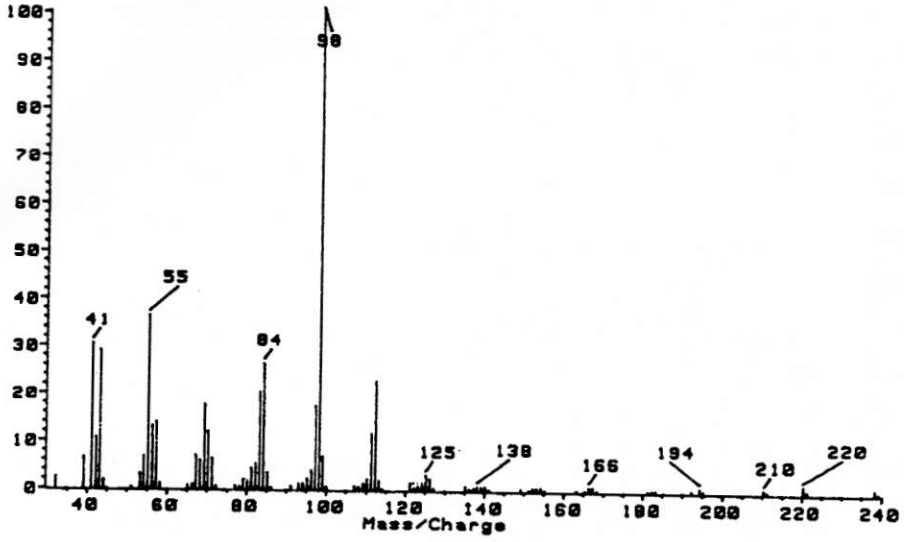


Figure A.3

Electron impact mass spectrum of TCB standard

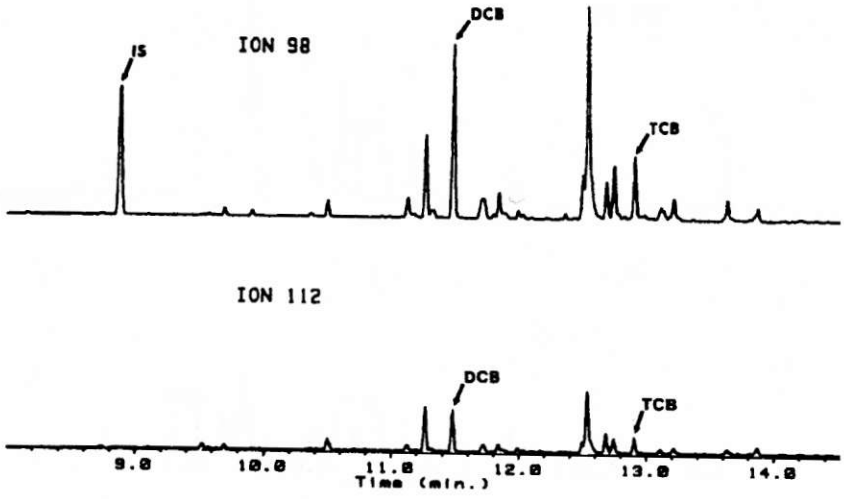


Figure A.4

Chromatogram of ions m/z 98 and m/z 112 for chicken irradiated with a dose of approximately 4.0 kGy (IS = Internal Standard)

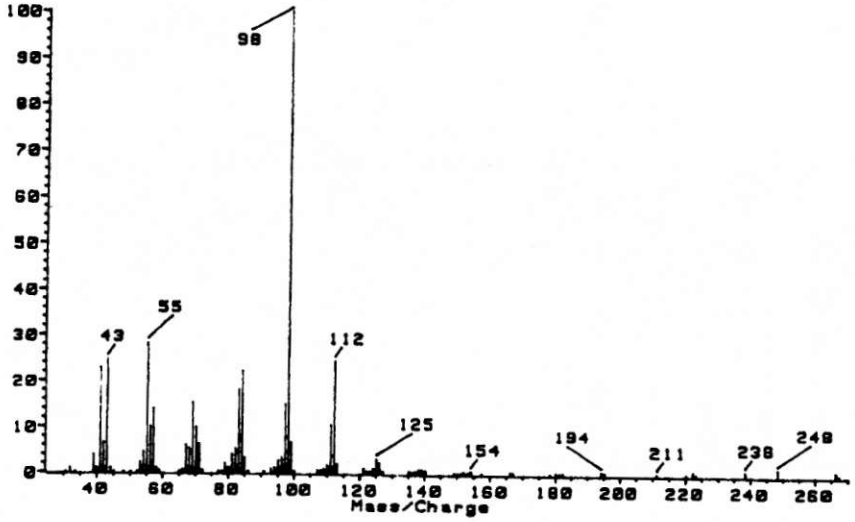


Figure A.5

Chromatogram of ions m/z 98 and m/z 112 for pork irradiated with a dose of approximately 3.0 kGy (IS = Internal Standard)

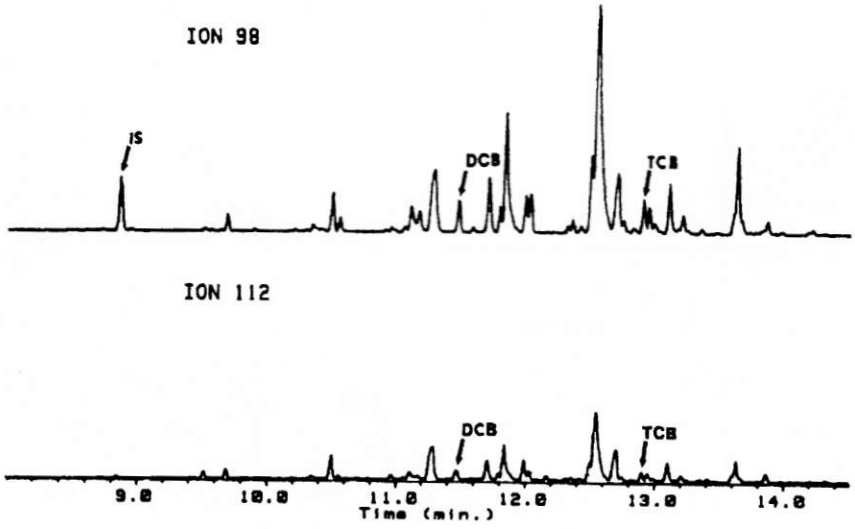
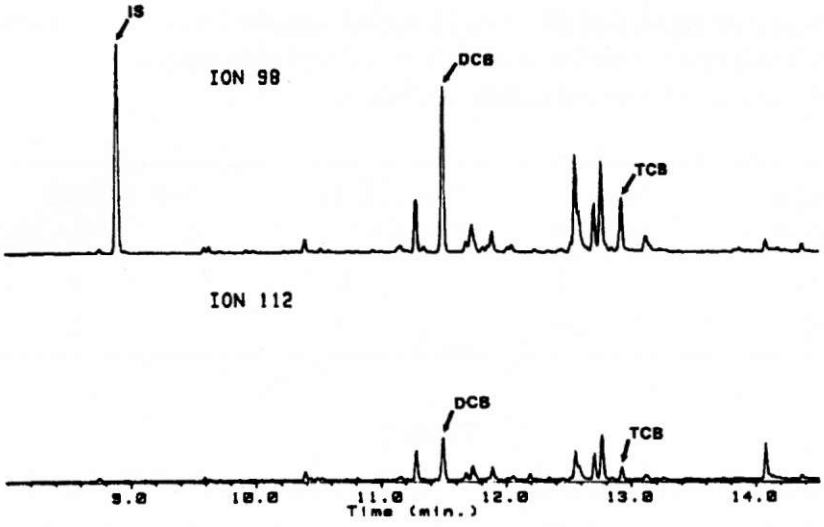


Figure A.6

Chromatogram of ions m/z 98 and m/z 112 for whole egg irradiated with a dose of approximately 3.0 kGy (IS = Internal Standard)



APPENDIX II

RESULTS OF COLLABORATIVE TRIALS

Table 1

Interlaboratory data of BCR collaborative blind trial

Five laboratories quantified DCB in 15 coded samples of chicken which were either not irradiated or irradiated with doses of approximately 0.5 kGy, 3.0 kGy or 5.0 kGy, one and six months after irradiation.

Time after irradiation	No. of samples	No. of false negatives[1]	No. of false positives[2]
1 month	74	0	0
6 months	60[3]	2	0

Table 2

Interlaboratory data for FAO/IAEA collaborative blind trial

Eleven laboratories used DCB and TCB to detect 9 coded samples of chicken, and liquid whole egg while 8 laboratories analysed pork. The samples were either unirradiated or given doses of 1.0 kGy or 3.0 kGy.

Sample	No. of samples	No. of false negatives	No. of false positives
Chicken	99	1[4]	0
Liquid whole egg	99	0	0
Pork	72	0	0

1 The false negatives were associated with samples given the 0.5kGy dose. False negatives are irradiated samples identified as unirradiated.

2 False positives are unirradiated samples identified as irradiated.

3 One laboratory did not provide results after the six months period.

4 Radiation-induced hydrocarbons were also not detectable in this sample so it was concluded that it had been miscoded.