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EVALUATION of MICROBIOLOGICAL METHODS of ANALYSIS for NATURAL MINERAL WATERS

Part 5

DETECTION OF SULPHITE-REDUCING ANAEROBES

COLLABORATIVE TRIAL

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The results of a collaborative trial of two methods for the detection of sporulated sulphite-reducing anaerobes (SRA) in natural mineral water are reported. Twenty-six UK laboratories participated in the trial to validate liquid enrichment and membrane filtration procedures prescribed by the International Organisation for Standardisation for use with potable waters. The results from this trial indicate that the liquid enrichment procedure is the better method for detection of sulphite-reducing anaerobes in natural mineral water. Where colony counts are desirable, the membrane filtration procedure is useful when used with tryptose sulphite agar. Data from the trial also demonstrated that the pasteurisation treatment given to samples prior to examination inactivated all vegetative cells of the autochthonous flora in the natural mineral water. Pre-trial investigations demonstrated poor growth of several Clostridium species under the trial conditions, therefore further studies are required to determine the efficacy of the ISO methods for the detection of a wide range of species of SRA.

The European Council of Ministers has adopted a Directive (80/777/EEC) on the Approximation of the Laws Relating to the Exploitation and Marketing of Natural Mineral Waters (NMW)[1]. This Directive was subsequently translated into legislation for England, Wales and Scotland[2] and also for Northern Ireland[3].

The Directive 80/777/EEC prescribes microbiological standards for NMW at source and specifies:

- the absence of coliforms and *Escherichia coli* in 250 ml at 30°C and 44.4°C
- the absence of faecal streptococci in 250 ml
- the absence of sporulated sulphite-reducing anaerobes (SRA) in 50 ml
- the absence of *Pseudomonas aeruginosa* in 250 ml
- the determination of total revivable colony count at 22°C and 37°C

Methods of analysis for NMW to enforce these standards are the microbiological methods for potable waters published by the International Organisation for Standardisation (ISO). These methods have been discussed in an EC Working Group: Such methods are assessed and validated within the UK by carrying out collaborative trials. Four trials of microbiological methods for NMW have already been carried out by MAFF, for the detection of *Escherichia coli* (Part I)[4], enumeration of total revivable colony count (Part II)[5], detection of *Pseudomonas aeruginosa* (Part III)[6] and detection of faecal streptococci[7]. This report describes the fifth trial organised by MAFF to validate microbiological methods of analysis for NMW and in particular for the demonstration of the absence of sporulated sulphite-reducing anaerobes as required by the Directive. The aims and objectives of these trials have been given previously[8].

Development of the Methods of Analysis Under Test

The following ISO methods were investigated:

- ISO 6461-1 :1986 Water Quality, Detection and enumeration of the spores of sulphite-reducing anaerobes (clostridia) - Part 1: Method by enrichment in liquid medium[9].

- ISO 6461-2:1986 Water Quality, Detection and enumeration of the spores of sulphite-reducing anaerobes (clostridia) - Part 2: Method by membrane filtration[10].

EC Directive 80/777/EEC requires demonstration of the absence of SRA in 50 ml of NMW. The ISO liquid enrichment procedure was therefore used only for the detection of SRA with no attempt at enumeration using a most probable number (MPN) technique.

Definition

For the purpose of this trial the following definition applies: Clostridia - sulphite-reducing, spore-forming, anaerobic micro-organisms which belong to the *Bacillaceae* family and the genus *Clostridium*.

COLLABORATIVE TRIAL ORGANISATION

Participants

A total of 26 UK laboratories participated in the trial, comprising 13 Public Analyst laboratories, 11 Public Health laboratories, one commercial laboratory and the MAFF Food Science Laboratory, Norwich.

Preparation and Distribution of Test Samples

Full details of the test sample preparation procedures are given in Appendix I. Protocols were distributed to participants several weeks in advance of the trial to allow for familiarisation with the methods.

NMW samples were prepared and cooled to *ca* 6°C on the morning of the day of distribution. They were transported in cool boxes (<10°C) and delivered to participating laboratories by overnight carrier to arrive the following morning. Participants were instructed to store samples at 4°C for 3 days prior to testing. Each laboratory received a total of 10 test samples; 5 × 50 ml samples of NMW for examination using the liquid enrichment technique and 5 × 100 ml samples of NMW for examination using the membrane filtration procedure. Each batch of 10 samples of NMW comprised of 3 sample types; samples were dispatched to participants as blind duplicates randomly coded. The laboratories were divided

into two groups of thirteen with one group receiving samples containing a background flora and the other receiving samples containing no background flora. The combination of samples for each group is shown below:

Group One (Laboratories 1-13)

Sample type A: 2 × 50ml and 2 × 100ml samples containing approximately 50 spores/50ml *Clostridium bifermentans* NCTC 506 (positive, high contamination level)

Sample type B: 2 × 50ml and 2 × 100ml samples containing approximately 10 spores/50ml *Clostridium bifermentans* NCTC 506 (positive, low contamination level)

Sample type C: 1 × 50ml and 1 × 100ml sample with no added *Clostridia* (negative sample)

Group Two (Laboratories 14-26)

Sample type A+: 2 × 50ml and 2 × 100ml samples containing approximately 50 spores/50ml *Clostridium bifermentans* NCTC 506 and 50 cells/50ml *Edwardsiella tarda* and *Proteus vulgaris*

(positive, high contamination level plus competitive flora)

Sample type B+: 2 × 50ml and 2 × 100ml samples containing approximately 10 spores/50ml *Clostridium bifermentans* NCTC 506 and 50 cells/50ml *Edwardsiella tarda* and *Proteus vulgaris* (positive, low contamination level plus competitive flora)

Sample type C+: 1 × 50ml and 1 × 100ml sample containing approximately 50 cells/50ml *Edwardsiella tarda* and *Proteus vulgaris* (negative sample)

METHODS

Participants were required to determine the presence/absence of SRAs' in all test samples provided using the methods described in Appendices II and III. Additionally, 10 samples of each sample type were examined at the Food Science Laboratory, Norwich, using the trial methods to ensure the homogeneity of all sample types.

The ISO method for membrane filtration gives the option of either incubating the filters on the surface of the agar in an anaerobic environment, or placing the filter "face-downwards" onto the agar and overlaying with molten isolation medium to produce an anaerobic environment. For the purpose of this trial, participants were instructed not to use the overlay option.

RESULTS

The data reported by all participants are shown in Tables 1-3 and summarised in Table 4.

Sample verification carried out at the Food Science Laboratory demonstrated that:

i) all Type A and A+ samples (high level positives) gave positive results using the membrane filtration and liquid enrichment procedures i.e. sulphite-reducing anaerobes present;

ii) all Type B samples (low level positives) were positive using the liquid enrichment procedure. However, using the membrane filtration technique with sulphite iron agar (SIA), only 7 of the 10 samples were positive and with tryptose sulphite agar (TSA) only 2 of the 10 samples were positive. For sample Type B+, 4 of the 10 samples tested using the liquid enrichment procedure were positive; using the membrane filtration technique with SIA, 9 of the 10 samples were positive and 6 of the 10 samples were positive using TSA.

iii) all Type C and C+ samples gave negative results using the membrane filtration and liquid enrichment procedures i.e. sulphite-reducing anaerobes absent.

DISCUSSION OF RESULTS

The pasteurisation treatment (75 ± 5°C for 15 minutes) applied to all samples before examination with either the liquid enrichment or membrane filtration procedures inactivated all vegetative cells present in the samples. No false positive results were reported.

Sample Verification

The results of sample verification experiments carried out at the Food Science Laboratory at the time of the trial were not consistent with those obtained either in pre-trial studies or reported by participants taking part in the trial. No false positive or false negative results were obtained from the samples inoculated with *Cl. bifermentans* at the higher level (50 spores/50 ml) during verification using the liquid enrichment technique. All samples containing the higher level of *Cl. bifermentans* when examined using the membrane filtration technique with isolation on SIA were positive, however, when TSA was used as the isolation medium, SRA were detected in only 80% of samples.

Using the liquid enrichment technique, SRA were detected in all samples containing *Cl. bifermentans* at the lower level (10 spores/50 ml) without competitive flora. However, for corresponding samples containing the low level

of *Cl. bifermentans* in the presence of a competitive flora, SRA were detected in only 40% of samples.

Using the membrane filtration procedure, in samples inoculated at the lower level without competitive flora, SRA were detected in 70% of samples using SIA as compared to 20% using TSA. Colony counts tended to be higher on SIA (mean count 2.3 cfu/50ml) than TSA (mean count 1.0 cfu/50ml).

Verification of the low level samples with competitive flora demonstrated similar results. SRA were detected in 90% of samples using SIA but in only 60% of samples using TSA. Again counts from the samples in which SRA were detected were higher using SIA (mean count 6.2 cfu/50ml) than TSA (mean count 2.7 cfu/50ml).

The reason for these anomalous results are not fully understood. However, the efficacy of the batch of media used for the sample verification must be questioned although in-house QC data did not indicate unsatisfactory results.

Liquid enrichment method

All samples containing *Cl. bifermentans* at a the higher level without competitive flora were correctly identified by participants. One laboratory reported a false negative result from one of the duplicate samples inoculated at the lower level in the presence of a competitive flora. No false positive results were reported.

Generally, participants reported that this method was easy to use but 2 laboratories expressed concern as to the safety of the procedure due to the reported risk of explosion of hermetically sealed broth cultures. One laboratory also stated that they found the medium difficult to prepare. Several laboratories reported blackening of the differential reinforced clostridial medium after only 24 h incubation. It may therefore be more useful to examine broths after 24 h to obtain an earlier result. Broths showing no blackening after 24 h incubation should still be re-incubated for a further 24h before a final result is reported.

In summary, use of the liquid enrichment procedure enabled correct identification of 259 of 260 samples (99%) with one false negative result reported. The liquid enrichment technique is suitable for detection of SRA in NMW and requires only simple apparatus. The main disadvantage of the method is the requirement for large volumes of double strength differential reinforced clostridial broth which can become expensive if large numbers of samples are required to be examined. Although the ISO method indicates that the procedure can be used for enumeration by using a most probable number (MPN) approach, the media, materials and time required to process samples would make this procedure impractical for the examination of large numbers of samples. The liquid

enrichment procedure is especially recommended for samples where low numbers of SRA are anticipated, such as in bottled waters.

Membrane filtration method

One false positive results was reported by a participant using the membrane filtration technique with SIA.

Results from samples inoculated with *Cl. bifermentans* at the higher level but with no competitive flora indicated that, using SIA, SRA were detected in only 50% of samples as compared to 85% using TSA. Similarly, for samples inoculated with *Cl. bifermentans* at the higher level in the presence of a competitive flora, SRA were detected in 77% of samples using SIA as compared to 92% using TSA.

For samples containing lower levels of *Cl. bifermentans* with and without competitive flora TSA again performed better than SIA. Sixty-nine percent of the low level samples with no competitive flora were correctly identified using TSA compared with only 42% using SIA. However, when a competitive flora was present, these figures increased to 85% and 73% respectively.

Statistical analysis of the colony counts from replicate samples revealed that there was no significant difference ($p<0.05$) between counts obtained using TSA and SIA from samples containing *Clostridium bifermentans* at both the higher and lower levels in the presence of competitive flora. However, colony counts obtained from the positive samples which contained no competitive flora were significantly higher ($p<0.05$) on TSA than SIA. There is no apparent reason why TSA should perform significantly better than SIA in the absence of competitive flora since when a competitive flora is present it is killed during the pasteurisation treatment applied prior to examination and thus should not affect the performance of the media.

One laboratory reported that the anaerobic jar used for incubation of the membrane filters on the isolation media failed, therefore no growth was obtained from any samples. This failure emphasises the importance of using well maintained anaerobic systems. A number of laboratories reported that the colonies produced on the membrane filters were grey, rather than black, as specified in the ISO standard. It was also noted during pre-trial studies that colony colour changed from black to grey when plates were left at ambient temperature in an aerobic atmosphere for prolonged periods (> 3h)

In summary, use of the membrane filtration procedure resulted in detection of SRA in a higher percentage of samples when TSA was used as the isolation medium as opposed to SIA. Although not always significantly different, the colony counts obtained using TSA tended to be higher than those from SIA. The

main advantage of the membrane filtration technique is that it gives an indication of the level of contamination with SRA and is suitable for processing large numbers of samples negating the need for large quantities of liquid media.

CONCLUSION

The results of this trial indicate that the liquid enrichment technique is more suitable for detection of SRA in natural mineral water than the membrane filtration method. However, when colony counts are desirable, the membrane filtration method is valuable but should be used it in conjunction with TSA and not SIA. Even with TSA some false negative results may be obtained. The efficacy of the pasteurisation treatment prescribed in the ISO standards was found to be satisfactory. Results also indicate there is value in examining the differential reinforced clostridial medium after 24 h incubation and re-incubating broths with no blackening for a further 24 h to allow possible earlier reporting of presumptive positive samples.

Importantly, pre-trial studies indicated that not all *Clostridia* spp. were able to be recovered on the media prescribed in the trial methods. Further work is required to determine the extent of this problem.

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10. International Organisation for Standardisation, 1986, Standard No. 6461-2 Water Quality: Detection and enumeration of the spores of sulphite-reducing anaerobes (clostridia) - Part 2: Method by membrane filtration.

APPENDIX I

Sample preparation

Water for artificial inoculation

Samples of commercially available bottled NMW were purchased on several occasions prior to the trial from a retail outlet and examined for the presence of sulphite-reducing anaerobes (SRA) using both the liquid enrichment and membrane filtration techniques. Autochthonous flora were present in the source but no SRA were detected on any occasion. For the purposes of the trial, the source was considered to be suitable for artificial inoculation.

Selection of organisms for artificial inoculation of samples

In order to fully validate the methods under test, several organisms were considered for use as competitive flora. Ideally, a range of samples were required containing not only typical strains of SRA but additionally organisms which may give similar reactions to SRA under the trial conditions but which are not members of the genus *Clostridium*.

A number of isolates from culture collections were examined for their reactions using the methods prescribed for the trial; these included *Edwardsiella tarda* NCTC 10396, *Bacillus cereus* NCDO 1771, *Bacillus subtilis* NCDO 1769, *Proteus vulgaris* NCDO 1882 and *Citrobacter freundii* NCDO 1484. The *Bacillus* spp. were selected because, as spore-formers, they are the organisms most likely to survive the pasteurisation step and thus challenge the performance of the isolation media. The other isolates were known sulphite-reducers. Neither of the *Bacillus* spp. were able to reduce sulphite under the trial conditions and were subsequently not included in trial samples. Of the remaining organisms, *Ed. tarda* and *Pr. vulgaris* closely simulated the reactions of SRA in the trial media; both species were included as competitive flora in 50% of trial samples at levels of 50 cells per 50 ml of NMW each.

A number of *Clostridium* spp. were evaluated for suitability as the SRA to be included in the NMW samples. These included *Cl. perfringens* NCTC 528, *Cl. sporogenes* NCTC 532, *Cl. novyi* NCTC 9746, *Cl. chauvoei* NCTC 8070, *Cl. bifermentans* NCTC 506 and *Cl. putrificum* NCTC 4718. Of these, *Cl. perfringens* gave very poor spore yields and *Cl. sporogenes* failed to grow in/on any of the trial media, neither of these were therefore suitable for inclusion in the trial. Of the remaining species, *Cl. bifermentans* produced the most typical growth in all media and when grown on blood agar base (Oxoid) under anaerobic conditions, produced large numbers of spores which were harvested in filter sterilised NMW and held at 4°C for 6 weeks. This ageing process was found to

be critical to the production of an homogeneous spore suspension from which vegetative cell growth could be instigated and reproducible cell counts obtained after pasteurisation.

Pre-trial studies indicated that *Cl. bifermentans, Pr. vulgaris* and *Ed. tarda,* at levels of 50 spores/cells per 50ml NMW, were stable in NMW for at least one week. Therefore, 7 litre volumes of the required sample types (see below) were prepared using appropriate dilutions of the aged *Cl. bifermentans* spore suspension and dilutions of nutrient broth (Oxoid) cultures (18 hours/30°C) of *Pr. vulgaris* and *Ed. tarda.*

After thorough mixing, trial samples were prepared by sub-sampling the 7 litre volumes of NMW and aseptically dispensing 50 ml (for liquid enrichment examinations) or 100 ml (for membrane filtration examinations) into sterile plastic screw top bottles (Sterilin) to produce the trial samples.

Participants were divided into two groups. Identical samples were examined by all participants except that Group 1 samples did not contain a competitive flora whereas Group 2 samples did.

APPENDIX II

DETECTION OF SULPHITE-REDUCING ANAEROBES (CLOSTRIDIA)

METHOD I

DETECTION BY ENRICHMENT IN A LIQUID MEDIUM

1. SCOPE & FIELD OF APPLICATION

The spores of sulphite-reducing anaerobes are widespread in the environment. They are present in human and animal faecal material, in waste water and soil. The spores survive in water for long periods of time and may thus give an indication of remote or intermittent pollution. This protocol specifies a method for the detection of the spores of sulphite-reducing anaerobes (clostridia) in natural mineral water by enrichment in a liquid medium.

2. DEFINITION

For the purpose of this protocol, the following definition of sulphite-reducing anaerobes applies: spore-forming, anaerobic micro-organisms which belong to the *Bacillaceae* family and the genus *Clostridium*, and which reduce sulphite under the test conditions.

3. PRINCIPLE

The detection of spores of sulphite-reducing anaerobes (*clostridia*) in 50ml of natural mineral water is based on the following steps.

3.1 Selection of Spores

Selection of spores in the sample by heating for a period of time sufficient to destroy vegetative bacteria.

3.2 Enrichment Culture

Detection of spores of sulphite-reducing anaerobes by inoculating a 50 ml volume of the sample into liquid enrichment media, followed by incubation at 37 ± 1°C for 44 ± 4 h in anaerobic conditions.

4. CULTURE MEDIA

4.1 Basic materials

In order to improve the reproducibility of results, it is recommended that for the preparation of diluents and culture media, dehydrated basic components or complete dehydrated media be used. Commercially prepared reagents may also be used. The manufacturer's instructions shall be rigorously followed.

All chemicals and reagents shall be of recognised analytical quality.

Water used shall be distilled or de-ionised and free from substances which may inhibit the growth of micro-organisms under the test conditions.

Measurements of pH shall be made using a pH meter, measurements being referred to a temperature of 25°C.

4.2 Differential Reinforced Clostridial Medium (DRCM) (not commercially available)

4.2.1 Single Strength Basal Medium

Peptone	10.0 g
Meat extract	10.0 g
Yeast extract	1.5 g
Starch	1.0 g
Hydrated sodium acetate	5.0 g
Glucose	1.0 g
L-Cysteine-hydrochloride	0.5 g
Water	up to 1000 ml

Preparation : Mix the peptone, meat extract, sodium acetate and yeast extract with 800 ml of water. With the remaining 200 ml of distilled water, prepare a starch solution as follows: mix the starch in a little cold water to form a paste. Heat the rest of the water to boiling point and slowly add it to the paste with constant stirring. Then add this starch solution to the first mixture and heat to boiling point until it dissolves. Finally, add the glucose and L-cysteine hydrochloride, and completely dissolve. Adjust the pH to 7.1 to 7.2 with 1 mol/l sodium hydroxide. Transfer 25 ml aliquots of the medium into screw-capped bottles. Sterilise in the autoclave at 121 ± 1°C for 15 min. Store between 2 to 8°C for up to 1 week (this medium is used for topping up, see **6.2**)

4.2.2 Double Strength Basal Medium

Prepare the double-strength medium as in **4.2.1** but reduce the volume of water by half. Transfer 50 ml aliquots of the medium into screw-capped bottles of 100 ml capacity, sterilise and store as above.

4.2.3 Sodium Sulphite (Na_2SO_3) Solution

Dissolve 4 g of anhydrous sodium sulphite in 100 ml of water to give a 4% w/v solution. Sterilise by filtration (0.22μm). Store at between 2 and 5°C for no more than 14 days.

4.2.4 Iron (III) Citrate ($C_6H_5O_7Fe$) Solution

Dissolve 7 g of iron (III) citrate in 100 ml of water to give a 7% w/v solution. Sterilise by filtration (0.22μm). Store at between 2 and 5°C for no more than 14 days.

4.2.5 Complete Medium

On the day of analysis, mix equal volumes of the solution of sodium sulphite and iron (III) citrate. Add 0.5 ml of the mixture to 25 ml of single strength medium which has been freshly heated for 15 minutes in a boiling water bath or steamer and cooled to room temperature. Add 2ml of the sodium sulphite/iron (III) citrate mixture to each 50 ml of double strength medium similarly treated.

5. APPARATUS AND GLASSWARE

Usual microbiological laboratory equipment including:

5.1 **Screw-cap Bottles** of boron silicate glass of *ca* 25ml and 100 ml capacity.

5.2 **Iron Wire**

5.3 **Incubator**, capable of being maintained at 37 ± 1°C.

5.4 **Water Bath**, maintained at 75 ± 5°C.

5.5 **Boiling water bath or steamer**

6. PROCEDURE

6.1 Selection of Spores

Heat the water sample to 75 ± 5°C in the water-bath. Simultaneously heat a similar water sample. Check the temperature of this control sample with a thermometer. Maintain the sample at 75 ± 5°C for 15 min.

6.2 Inoculation and Incubation

Add 50 ml of sample (**6.1**) to a 100 ml screw-cap bottle containing 50 ml of the double-strength complete medium (**4.2.5**).

If necessary, top up all the bottles with the single strength complete medium (**4.2.5**) to bring the volume of liquid level with the neck and to ensure that only a very small volume of air remains. Seal the bottles hermetically or incubate under anaerobic conditions at 37 ± 1°C for 44 ± 4 h.

SAFETY NOTE: Large volumes of culture media in hermetically sealed glass bottles may explode due to gas production. The addition of iron wire, heated to redness and placed into the medium before inoculation, may aid anaerobiosis.

7. EXPRESSION OF RESULTS

Bottles in which blackening is observed, as a result of the reduction of sulphite and the precipitation of iron (II) sulphide, shall be regarded as positive.

Express the results as presence (+) or absence (-) of sulphite-reducing anaerobes in 50 ml natural mineral water.

8. QUALITY CONTROL

The isolation and confirmation procedures should be assessed by the inclusion of suitable control organisms as follows:-

Recommended controls: positive - *Clostridium perfringens* NCTC 8237

negative - *Escherichia coli* NCTC 9001

9. REFERENCES

9.1 European Standard (EN) 1993 - Water Quality Detection and enumeration of spores of sulphite-reducing anaerobes (clostridia). Part 1 - Method by enrichment in a liquid medium. EN 26461 - 1

METHOD II

DETECTION BY MEMBRANE FILTRATION

1. SCOPE & FIELD OF APPLICATION

The spores of sulphite-reducing anaerobes are widespread in the environment. They are present in human and animal faecal material, in waste water and soil. The spores survive in water for long periods of time and may thus give an indication of remote or intermittent pollution. This protocol specifies a method for the detection of the spores of sulphite-reducing anaerobes (*clostridia*) in natural mineral water by membrane filtration.

2. DEFINITION

For the purpose of this protocol the following definition of sulphite-reducing anaerobes applies: spore-forming, anaerobic micro-organisms which belong to the *Bacillaceae* family and the genus *Clostridium* and which develop at 37°C after 24 - 48 hours on a sulphite containing agar producing typical colonies.

3. PRINCIPLE

The detection of the spores of sulphite-reducing anaerobic bacteria in 50 ml volumes of natural mineral water is based on the destruction of vegetative cells by applying heat to the sample of water, followed by filtration through a membrane filter with a pore size which will retain spores. The filters are placed on one or more selective culture media containing sodium sulphite and iron salts and incubated at 37°C ± 1°C under anaerobic conditions for up to 48h. Typical colonies are black.

4. CULTURE MEDIA

4.1 Basic materials

In order to improve the reproducibility of results, it is recommended that for the preparation of diluents and culture media, dehydrated basic components or complete dehydrated media be used. Commercially prepared reagents may also be used. The manufacturer's instructions shall be rigorously followed.

All chemicals and reagents shall be of recognised analytical quality.

Water used shall be distilled or de-ionised and free from substances which may inhibit the growth of micro-organisms under the test conditions.

Measurements of pH shall be made using a pH meter, measurements being referred to a temperature of 25°C.

Use both the following media as directed:

4.2 Sulphite Iron Agar (not commercially available)

4.2.1 Basal Medium

Meat extract	3.0 g
Peptone	10.0 g
Sodium chloride	5.0 g
Agar	10 - 20 g (1)
Water	up to 1000 ml

(1) Depending on the gelling power of the agar

Preparation : Dissolve ingredients in the water by steaming. Adjust the pH to 7.6 ± 0.1 with 1 mol/l sodium hydroxide solution. Dispense into 180 ml amounts. Sterilise at 121°C in the autoclave for 20 min. Store in the refrigerator after solidifying.

4.2.2 Sodium Sulphite (Na_2SO_3) Solution

Dissolve 10 g sodium sulphite in 100 ml of water. Sterilise by filtration (0.22μm). Store for no longer than 2 weeks.

4.2.3 Iron (II) Sulphate ($FeSO_4$) Solution

Dissolve 8 g crystallised iron (II) sulphate in 100 ml of water. Sterilise by filtration (0.22μm).

4.2.4 Complete Medium

Immediately before use, melt the basal medium (**4.2.1**) and to each 180ml, add 10 ml of sodium sulphite solution and 2 ml of iron (II) sulphate solution. Pour the complete medium into Petri dishes to a depth of at least 3 mm and allow to set on a cool, horizontal surface.

NOTE : The sodium sulphite and the iron (II) sulphate should be added to the basal medium immediately prior to pouring agar plates.

4.3 Tryptose-sulphite Agar (Difco SFP Agar base 0811-01-8 or equiv.)

Tryptose	15.0 g
Soytone	5.0 g
Yeast extract	5.0 g
Sodium meta-bisulphite	1.0 g
Ammonium iron (III) citrate	1.0 g
Agar	10 - 20 g (1)
Water	up to 1000ml

(1) Depending on the gelling power of the agar

Preparation : Dissolve ingredients in the water by steaming. Adjust to pH 7.6±0.1 at 25°C. Sterilise the medium for 15 min at 121°C. Pour the medium into Petri dishes to a depth of at least 3 mm and allow to set on a cool, horizontal surface. The medium may be stored for a maximum of 2 weeks at 4 to 5°C.

5. APPARATUS AND GLASSWARE

Usual microbiological equipment including:

5.1 **Incubator**, capable of being maintained at 37 ± 1°C.

5.2 **Water bath**, capable of being maintained at 75°C ± 5°C.

5.3 **Membrane filter apparatus**, of a sufficient capacity to contain 50ml, with vacuum flask tubing, a moisture trap flask and a vacuum source.

5.4 **Sterile forceps**

5.5 **Sterile membrane filters**, with a pore size equal to or less than 0.22μm and approximately 47 mm in diameter.

6. PROCEDURE

6.1 Selection of Spores

Heat the water sample to 75 ± 5°C in the water-bath. Simultaneously heat a similar water sample. Check the temperature of this control sample with a thermometer. Maintain the sample at 75 ± 5°C for 15 min.

6.2 Inoculation and Incubation

6.2.1 After thorough mixing by shaking, from the 100 ml water sample, filter a 50ml volume of water. Remove the membrane with sterile forceps and place, face upwards, on the surface of sulphite iron agar, ensuring that no air bubbles are trapped under the filter. Without rinsing the filter apparatus, filter the second 50 ml volume through a clean membrane and place as above on the surface of tryptose sulphite agar. Incubate both agars in an anaerobic jar / anaerobic incubator at 37 ± 1°C for 20 ± 4 h and 44 ± 4 h.

NOTE - Funnels should be thoroughly rinsed in sterile distilled water and sterilised by autoclaving at 121°C for 15 minutes, and then cooled between **different** samples. (To avoid this, separate funnels may be used for each sample)

7. ENUMERATION OF CFU OF SULPHITE-REDUCING ANAEROBES

Consider the formation of black colonies as confirmation of the presence of sporulated, sulphite-reducing anaerobes. Count all black colonies after incubation for 24 and 48h. Express the results as number of cfu per 50 ml natural mineral water.

8. QUALITY CONTROL

The isolation and confirmation procedures should be assessed by the inclusion of suitable control organisms as follows:-

Recommended controls: positive - *Clostridium perfringens* NCTC 8237

negative - *Escherichia coli* NCTC 9001

9. REFERENCES

9.1 European Standard 1993 - Water Quality. Detection and enumeration of the spores of sulphite-reducing anaerobes (clostridia) Part 2 : Method by membrane filtration.

Table 1

Detection of sulphite-reducing anaerobes in NMW using the liquid enrichment technique

LAB CODE	SAMPLE TYPE		
	A	B	C
1	+/+	+/+	-
2	+/+	+/+	-
3	+/+	+/+	-
4	+/+	+/+	-
5	+/+	+/+	-
6	+/+	+/+	-
7	+/+	+/+	-
8	+/+	+/+	-
9	+/+	+/+	-
10	+/+	+/+	-
11	+/+	+/+	-
12	+/+	+/+	-
13	+/+	+/+	-
	A+	B+	C+
14	+/+	+/+	-
15	+/+	+/+	-
16	+/+	+/+	-
17	+/+	+/+	-
18	+/+	+/+	-
19	+/+	+/+	-
20	+/+	+/+	-
21	+/+	+/+	-
22	+/+	+/+	-
23	+/+	+/+	-
24	+/+	+/+	-
25	+/+	+/-	-
26	+/+	+/+	-
True Result	+	+	-

\+ sulphite-reducing anaerobes detected in 50 ml of natural mineral water

\- sulphite-reducing anaerobes not detected in 50 ml of natural mineral water

Sample Type A contained 50 spores of *Cl. bifermentans* per 50 ml of natural mineral water.

Sample Type B contained 10 spores of *Cl. bifermentans* per 50 ml of natural mineral water.

Sample Type C contained natural mineral water free from sulphite-reducing anaerobes.

Sample Type A+ contained 50 spores *Cl. bifermentans*, 50 cells *Pr. vulgaris* and *Ed. tarda* per 50 ml NMW

Sample Type B+ contained 10 spores *Cl. bifermentans*, 50 cells *Pr. vulgaris* and *Ed. tarda* per 50 ml NMW

Sample Type C+ contained 50 cells *Pr. vulgaris* and *Ed. tarda* per 50 ml NMW

Table 2

Colony counts of sulphite-reducing anaerobes in NMW using the membrane filtration technique and sulphite iron agar

LAB CODE	SAMPLE TYPE		
	A	B	C
1	0/0	0/0	0
2	0/0	0/0	0
3	0/0	0/0	0
4	6/22	2/3	0
5	1/2	2/2	0
6	25/57	4/0	0
7	5/10	6/4	0
8	0/0	0/0	0
9	90/60	12/10	0
10	24/6	0/0	0
11	0/0	0/0	1
12	0/0	0/0	0
13	0/12	4/6	0
	A+	**B+**	**C+**
14	37/31	4/3	0
15	0/0	0/0	0
16	0/0	0/0	0
17	11/23	0/6	0
18	66/58	16/10	0
19	63/62	10/10	0
20	32/21	7/12	0
21	64/74	8/4	0
22	90/60	12/10	0
23	29/40	10/15	0
24	74/47	4/6	0
25	0/0	0/0	0
26	65/55	12/7	0
True Result	50	10	0

\+ sulphite-reducing anaerobes detected in 50 ml of natural mineral water

\- sulphite-reducing anaerobes not detected in 50 ml of natural mineral water

Sample Type A contained 50 spores of *Cl. bifermentans* per 50 ml of natural mineral water.

Sample Type B contained 10 spores of *Cl. bifermentans* per 50 ml of natural mineral water.

Sample Type C contained natural mineral water free from sulphite-reducing anaerobes.

Sample Type A+ contained 50 spores *Cl. bifermentans*, 50 cells *Pr. vulgaris* and *Ed. tarda* per 50 ml NMW

Sample Type B+ contained 10 spores *Cl. bifermentans*, 50 cells *Pr. vulgaris* and *Ed. tarda* per 50 ml NMW

Sample Type C+ contained 50 cells *Pr. vulgaris* and *Ed. tarda* per 50 ml NMW

Table 3

Colony counts of sulphite-reducing anaerobes in NMW using the membrane filtration technique and tryptose sulphite agar

LAB CODE	SAMPLE TYPE		
	A	**B**	**C**
1	12/5	1/2	0
2	0/0	0/0	0
3	34/30	6/12	0
4	33/32	6/2	0
5	18/16	1/2	0
6	47/17	13/3	0
7	64/34	10/5	0
8	19/45	0/0	0
9	65/41	6/13	0
10	35/9	0/0	0
11	0/0	0/0	0
12	2/1	1/3	0
13	6/17	2/21	0
	A+	**B+**	**C+**
14	21/13	5/5	0
15	0/0	0/0	0
16	2/10	5/0	0
17	15/15	3/3	0
18	53/69	14/14	0
19	41/56	10/4	0
20	44/50	10/7	0
21	62/27	8/16	0
22	42/30	9/6	0
23	9/5	11/17	0
24	67/64	19/11	0
25	69/50	12/16	0
26	17/38	2/0	0
True Result	50	10	0

\+ sulphite-reducing anaerobes detected in 50 ml of natural mineral water

\- sulphite-reducing anaerobes not detected in 50 ml of natural mineral water

Sample Type A contained 50 spores of *Cl. bifermentans* per 50 ml of natural mineral water.

Sample Type B contained 10 spores of *Cl. bifermentans* per 50 ml of natural mineral water.

Sample Type C contained natural mineral water free from sulphite-reducing anaerobes.

Sample Type A+ contained 50 spores *Cl. bifermentans*, 50 cells *Pr. vulgaris* and *Ed. tarda* per 50 ml NMW

Sample Type B+ contained 10 spores *Cl. bifermentans*, 50 cells *Pr. vulgaris* and *Ed. tarda* per 50 ml NMW

Sample Type C+ contained 50 cells *Pr. vulgaris* and *Ed. tarda* per 50 ml NMW

Table 4

Summary of results for the detection of sulphite-reducing anaerobes in NMW

Method of Examination	A		A+		B		B+		C		C+	
	Samples tested	% positive	Samples tested	% positive	Samples tested	% positive	Samples tested	% positive	Samples tested	% positive	Samples tested	% positive
Liquid enrichment	26	100	26	100	26	100	26	96.2	13	0	13	0
Sulphite iron agar #	26	50	26	76.9	26	42.3	26	73.1	13	0	13	0
Tryptose sulphite agar #	26	84.6	26	92.3	26	69.2	26	84.6	13	0	13	0

Key to sample types

A 50 spores *C. bifermentans* per 50 ml NMW
A+ 50 spores *C. bifermentans*, 50 cells *Ed. tarda*, 50 cells *Pr. vulgaris* per 50 ml NMW
B 10 spores *C. bifermentans* per 50 ml NMW
B+ 10 spores *C. bifermentans,* 50 cells *Ed. tarda*, 50 cells *Pr. vulgaris* per 50 ml NMW per 50 ml NMW
C NMW with no added bacteria
C+ 50 cells *Ed. tarda*, 50 cells *Pr. vulgaris* per 50 ml NMW
membrane filtration method

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MAFF VALIDATED METHODS FOR THE ANALYSIS OF FOODSTUFFS

DIETARY FIBRE: ENGLYST PROCEDURE FOR DETERMINATION OF DIETARY FIBRE AS NON-STARCH POLYSACCHARIDES:

MEASUREMENT OF CONSTITUENT SUGARS BY GAS–LIQUID CHROMATOGRAPHY

V. 39

Correspondence on this method may be sent to Hans Englyst, Dunn Clinical Nutrition Centre, Hills Road, Cambridge, CB2 2DH

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 7UQ

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, with respect to the sample preparation.

1. SCOPE AND FIELD OF APPLICATION

The Englyst procedure determines total, soluble and insoluble non-starch polysaccharides (NSP) in plant foods and food products. The cell-wall material naturally present in plants is the common characteristic of the foods that comprise a high-fibre diet and approximately 90% of this material is NSP. The specific measurement of the NSP content of unfortified plant foods provides a good marker for endogenous plant cell-wall material and, therefore, for a high-fibre diet (i.e. a diet rich in plant cell-wall material, minerals, vitamins and antioxidants but low in fat and sodium).

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The EC Scientific Committee for Food have been considering the definition of dietary fibre for food labelling and have reported (**9.1**):

"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."

NSP values will aid the consumer in choosing the type of naturally high-fibre diet for which health benefits have been shown, and which is recommended in national dietary guidelines.

The procedure is widely used in research, and it is one of the methods used for food labelling for dietary fibre within the EC.

NSP values are used in European nutritional databases and are reported as dietary fibre in food tables (e.g. **9.2 -9.4**).

2. DEFINITION

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

3. PRINCIPLE

The Englyst procedure (**9.5 - 9.8**) measures non-starch polysaccharides (NSP), using enzymic-chemical methods, and has evolved from the principles laid down by McCance and Widdowson, and later by Southgate (**9.9**). In the procedure, starch is dispersed and then hydrolysed enzymically. The NSP are precipitated with alcohol and hydrolysed by sulphuric acid, releasing neutral sugars, which are measured individually as alditol acetates by GLC, and uronic acids, which are measured by colorimetry (**9.6, 9.7**). Values for total, soluble and insoluble NSP as the sum of individual neutral sugars and uronic acids may be obtained within 1.5 working days.

(When details of the component sugars are not required, the use of a colorimetric end-point for both neutral sugars and uronic acids allows NSP values to be obtained within an 8 hr. working day (**9.7**). Agreement is good between the GLC and colorimetric assay, which is suitable for automation (**9.5**))

The Englyst procedure for the measurement of NSP by GLC is summarised in Appendix I.

4. REAGENTS

All the reagents marked with an asterisk (*), together with four reference samples, are available in Kit form from the MRC Dunn Clinical Nutrition Centre.

High-purity reagents and distilled water, or water of an equivalent purity, should be used throughout the method.

4.1 **Acetic acid**, glacial

4.2 **Acetic anhydride**

4.3 **Acetone**

4.4 **Ammonium hydroxide**, 12 mol/l

4.5 **Ammonium hydroxide/sodium borohydride solution**. A solution of ammonium hydroxide, 6 mol/l, containing 200 mg of sodium borohydride, $NaBH_4$, per ml. Prepare immediately before use.

4.6 **Benzoic acid**, 50% saturated: prepare by diluting saturated benzoic acid 1:1 v/v with water.

4.7 **Bromophenol blue**, 0.04% w/v.

4.8 **3,5-Dimethylphenol**, dissolve 0.1 g of 3,5-dimethylphenol, $(CH_3)_2C_6H_3OH$, in 100 ml of glacial acetic acid (**4.1**).

4.9 **Dimethyl sulphoxide**

4.10 **Enzyme solution I**, take 2.5 ml of heat-stable amylase (EC. 3.2.1.1; Termamyl, 300 KNU/g Novo Nordisk*) and make to 200 ml with the pre-equilibrated sodium acetate buffer (**4.23**), mix, and keep it in a 50°C water-bath (**5.4**). Prepare the solution immediately before use.

4.11 **Enzyme solution II**, take 1.2 g of pancreatin (Pancrex V powder from Paynes & Byrne Ltd, Greenford, Middlesex, UK or Sigma cat. no. P1500*) into a 50 ml tube, add 12 ml of water, vortex-mix initially and then mix for 10 min. with a magnetic stirrer. Vortex-mix again, then centrifuge for 10 min. Take 10 ml of the (cloudy) supernatant, add 2.5 ml of pullulanase (EC 3.2.1.41; 200 PNU/g Novo Nordisk*) and vortex-mix. Prepare the solution immediately before use and keep it at room temperature.

4.12 **Ethanol, absolute**.

4.13 **Ethanol, absolute**, acidified. Add 1 ml of hydrochloric acid, 5 mol/l (**4.18**), per litre of ethanol (**4.12**).

4.14 **Ethanol, 85% v/v, acidified**. Add 150 ml of water to 1 ml of hydrochloric acid, 5 mol/l (**4.18**), and make to 1 litre with ethanol (**4.12**).

4.15 **Glass balls**, 2.5 – 3.5 mm diameter (Merck cat. no. 33212).

4.16 **GLC internal standard solution**, 1 mg/ml. Weigh 500 mg of allose (dried to constant weight under reduced pressure with phosphorus pentoxide) to the nearest 0.1 mg. Make to 500 ml with 50% saturated benzoic acid (**4.6**) to give a 1 mg/ml solution. The solution is stable for several months at room temperature.*

4.17 **GLC stock sugar mixture**, weigh (all sugars dried to constant weight under reduced pressure with phosphorus pentoxide), to the nearest 1 mg, 0.52 g of rhamnose, 0.48 g of fucose, 4.75 g of arabinose, 4.45 g of xylose, 2.3 g of mannose, 2.82 g of galactose, 9.4 g of glucose and 2.79 g (3.05 g of the monohydrate) of galacturonic acid. Place them in a 1 litre calibrated flask and dilute to volume with 50% saturated benzoic acid (**4.6**). The solution is stable at room temperature for several months.*

4.18 **Hydrochloric acid**, 5 mol/l.

4.19 **1-Methylimidazole**

4.20 **Octan-2-ol**

4.21 **Potassium hydroxide**, 7.5 mol/l.

4.22 **Sand, acid-washed**, 50–100 mesh (Merck cat. no. 33094).

4.23 **Sodium acetate buffer**, 0.1 mol/l, pH 5.2: adjust sodium acetate, 0.1 mol/l (**4.23.1**), to pH 5.2 with acetic acid, 0.1 mol/l (**4.23.2**). To stabilise and activate enzymes, add 4 ml of calcium chloride, 1 mol/l (**4.23.3**), to 1 litre of buffer.

4.23.1 **Sodium acetate**, 0.1 mol/l: dissolve 13.6 g of sodium acetate trihydrate, $CH_3COONa.3H_2O$, and make to a final volume of 1 litre with water.

4.23.2 **Acetic acid**, 0.1 mol/l.

4.23.3 **Calcium chloride**, 1 mol/l.

4.24 **Sodium chloride/boric acid**, dissolve 2 g of sodium chloride, NaCl, and 3 g of boric acid, H_3BO_3, in 100 ml of water.

4.25 **Sodium phosphate buffer**, 0.2 mol/l, pH 7: adjust Na_2HPO_4, 0.2 mol/l (**4.25.1**), to pH 7 with NaH_2PO_4, 0.2 mol/l (**4.25.2**). Prepare buffer on day of use.

4.25.1 **Disodium hydrogen phosphate**, 0.2 mol/l. Stable for several months at room temperature.

4.25.2 Sodium dihydrogen phosphate, 0.2 mol/l. Stable for several months at room temperature.

4.26 Sulphuric acid, concentrated.

4.27 Sulphuric acid, 2 mol/l. Add 5 ml of sulphuric acid, 12 mol/l (**4.28**) to 25 ml of water. Cool to room temperature before use.

4.28 Sulphuric acid, 12 mol/l. This may be obtained as sulphuric acid, 72% w/w (Merck cat. no. 19321 6Y).

4.29 Sulphuric acid, 2.4 mol/l. Add 5 ml of sulphuric acid, 12 mol/l (**4.28**), to 20 ml of water. Cool to room temperature before use.

5. APPARATUS

5.1 Balance, accurate to 0.1 mg.

5.2 Centrifuge, capable of exerting 1500 *g*.

5.3 Centrifuge tubes, glass centrifuge tubes of 50-60 ml capacity (Anachem cat. no. 45212-50), fitted with Teflon-lined screw tops (24 tubes are suitable for a batch). Make a mark at 50 ml.

5.4 Water-baths, one capable of maintaining 100°C; one capable of maintaining temperatures in the range 35°C to 70°C. Each bath should be of such a capacity that there is no significant change in temperature when a rack containing all the tubes (**5.3**) is placed in it.

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

5.6 Vortex-mixer

5.7 GLC chromatograph, fitted with flame ionisation detector and, preferably, auto-injector and computing integrator or chromatography data handling system.

5.8 GLC column, capable of separating alditol acetates; e.g. Supelco SP-2380 wide-bore capillary column (30 m × 0.53 mm id: Supelco cat. no. 2-5319) or SGE BPX70 wide-bore capillary column (30 m × 0.75 mm id: SGE part. no.054620).

5.9 Heating block, capable of maintaining 40°C and 70°C.

5.10 Fan oven, capable of maintaining 80°C.

6. PROCEDURE

6.1 Pre-treatment of samples

All samples should be finely divided so that representative sub-samples may be taken. Foods with a low water content (<10%) may be milled, and foods with a higher water content may be homogenised wet or milled after freeze-drying.

6.2 Test samples and sample weights

6.2.1 Test samples

Two portions, (a) and (b), of each test sample are required to obtain separate values for total, insoluble and soluble NSP. Portion (a) is used to measure total NSP; portion (b) is used to measure insoluble NSP. Soluble NSP is determined as the difference. The two portions are treated identically throughout the procedure, except for their separate treatment in steps 6.5 and 6.6.

6.2.2 Sample weight

Weigh (**5.1**), to the nearest 0.1 mg, between 50 and 1000 mg depending on the water and NSP content of the sample (to give not more than 300 mg of dry matter; e.g. 300 mg is adequate for most dried foods but use 100 mg for bran and purified fibre preparations) into 50-60 ml screw-top glass tubes (**5.3**). Add 300 (±20) mg of acid-washed sand (**4.22**) and approximately 15 glass balls (**4.15**) to each tube. If the sample is dry (90 to 100% dry matter) and contains less than 10% fat, proceed to step 6.4; otherwise, go to step **6.3**. (It is recommended that all analyses are carried out in duplicate.)

6.3 Fat extraction/drying

Add 40 ml of acetone (**4.3**), cap the tubes and mix several times over a 30 min. period. Centrifuge (**5.2**) at 1000 *g* for 10 min. to obtain a clear supernatant liquid and remove by aspiration as much of the supernatant liquid as possible without disturbing the residue. Discard the supernatant. Vortex-mix vigorously to ensure that the residue is dispersed thinly around the bottom 5 cm of the tube.

Place the tubes in a pan of water at 75°C in a fume-cupboard. Remove the tubes singly and vortex-mix vigorously at frequent intervals until the tubes and residues are dry. (Note: an evaporator may be used, see **6.5.4**.)

6.4 Dispersion and enzymic hydrolysis

6.4.1 Treatment with dimethyl sulphoxide (DMSO)

Pre-equilibrate sufficient acetate buffer (**4.23**) at 50°C (8 ml required per sample).

Add 2 ml of DMSO (**4.9**) to the dry sample, cap the tube, and immediately mix the contents using a vortex-mixer (**5.6**). It is essential that all the sample is wetted and no material is encapsulated or adhering firmly to the tube wall before proceeding. When DMSO has been added and mixed in all the tubes, vortex-mix a further three or four times during a 5 min. period.

Vortex-mix and immediately place two of the tubes into a boiling water-bath (**5.4**). Remove them after 20 seconds, vortex-mix and replace them in the boiling water-bath. Repeat this for subsequent pairs of tubes until all the tubes are in the bath; leave them there for 30 min. from that time. During this period, prepare enzyme solutions I (**4.10**) and II (**4.11**). The volumes given are suitable for 24 tubes.

6.4.2 Treatment with enzymes

Remove one tube at a time, vortex-mix, uncap and immediately add, by positive displacement, 8 ml of enzyme solution I (**4.10**), cap the tube, vortex-mix thoroughly, ensuring that no material adheres firmly to the tube wall, and replace it in the boiling water-bath. Leave the tubes there for 10 min., timed from the last addition of enzyme.

Transfer the rack of tubes to the 50°C water-bath. After 3 min., add 0.5 ml of enzyme solution II (**4.11**) to each tube and mix the contents thoroughly to aid distribution of the enzyme throughout the sample. Replace the tubes in the 50°C water-bath and leave them there for 30 min. Transfer the rack of tubes to the boiling water-bath and leave them there for 10 min.

6.5 Precipitation and washing of the residue for measurement of total NSP: test sample portion (a) only

6.5.1 Cool the sample by placing in ice–water. Add 0.15 ml of hydrochloric acid, 5 mol/l (**4.18**), and vortex-mix thoroughly two or three times during a 5 min. period, with tubes being replaced in the ice–water. Add 40 ml of acidified ethanol, absolute (**4.13**), to give a final concentration of 80% v/v. Mix well by repeated inversion, then leave in ice–water for 30 min. Centrifuge (**5.2**) at 1500 *g* for 10 min. to obtain a clear supernatant liquid. Remove by decanting or aspiration as much of the supernatant liquid as possible, without disturbing the residue, and discard the liquid.

6.5.2 Add approximately 10 ml of acidified ethanol, 85% (**4.14**), to the residue and vortex-mix. Make to 50 ml with acidified ethanol, 85% (**4.14**), cap the tube and mix thoroughly by repeated inversion. Centrifuge and remove the supernatant liquid as above. Repeat this stage using 50 ml of ethanol, absolute (**4.12**).

6.5.3 Add 30 ml of acetone (**4.3**) to the residue and vortex-mix thoroughly to form a suspension. Centrifuge and remove the supernatant liquid as in step **6.5.1**.

6.5.4 Vortex-mix vigorously to ensure that the residue is dispersed thinly around the bottom 5 cm of the tube. Place the rack of tubes in a pan of water at 70 to 75°C in a fume-cupboard. Remove the tubes singly and vortex-mix vigorously at frequent intervals, to ensure that the residue in each tube is finely divided, until the tube and residue appear dry. Place the rack of tubes in a fan oven (**5.10**) at 80°C for 10 min. to remove any traces of acetone. It is essential that the residues and tubes are completely free of acetone. (Instead of using the fume-cupboard/drying oven, it is convenient to dry the residue in an evaporator (e.g. TurboVap, Zymark Ltd, Cheshire, UK). After 1 min. mix again and replace the tubes in the apparatus. Repeat until the residue is free of lumps and the tubes and residues are free of acetone.)

6.6 Extraction and washing of the residue for measurement of insoluble NSP: test sample portion (b) only.

6.6.1 After the treatment with enzymes in step 6.4, add 40 ml of sodium phosphate buffer (**4.25**). Place the capped tubes in the boiling water-bath and leave them there for 30 min. Mix continuously or a minimum of three times during this period. Remove the tubes and equilibrate to room temperature in water. Centrifuge and remove the supernatant liquid as in step **6.5.1**.

6.6.2 Add approximately 10 ml of water and vortex-mix. Make to 50 ml with water and mix well by repeated inversion. Centrifuge and remove the supernatant liquid as above. Repeat this stage using 50 ml of ethanol, absolute.

6.6.3 Proceed as described for step **6.5.3** and step **6.5.4.**

6.7 Acid hydrolysis of the residue from steps 6.5 and 6.6

6.7.1 Add 5 ml of sulphuric acid, 12 mol/l (**4.28**), to one tube and immediately vortex-mix vigorously; ensure that all the material is wetted. Repeat this for each tube in turn. Once the acid has been added to all the tubes, vortex-mix again and place the tubes into a water-bath at 35°C. Leave the tubes for 30 min. with vigorous vortex-mixing after 5, 10 and 20 min. to disperse the cellulose. To each tube in turn, add 25 ml of water rapidly from a positive displacement device and vortex-mix. Place all the tubes into a boiling water-bath (**5.4**) and leave them there for 1 hr., timed from when boiling recommences; mix after 10 min. Remove all the tubes together and cool rapidly in water.

7. MEASUREMENT OF CONSTITUENT NEUTRAL SUGARS BY GAS-LIQUID CHROMATOGRAPHY AND URONIC ACIDS BY COLORIMETRY

7.1 Standard sugar mixture for GLC calibration

Mix 1.0 ml of the GLC stock sugar mixture (**4.17**) with 5 ml of sulphuric acid, 2.4 mol/l (**4.29**). Treat 2 × 1 ml of this standard sugar mixture for calibration of GLC in parallel with the hydrolysates from step **6.7** of the procedure.

7.2 Preparation of alditol acetate derivatives for GLC

Prepare sufficient (0.2 ml is required for each tube) of the ammonium hydroxide/sodium borohydride solution (**4.5**).

Add 0.50 ml of GLC internal standard (1 mg allose/ml (**4.16**)) to 1 ml of the cooled hydrolysates from step **6.7** and to 2 × 1 ml of the standard sugar mixture from step **7.1**, vortex-mix.

Place the tubes in ice–water, add 0.4 ml of ammonium hydroxide, 12 mol/l (**4.4**), and vortex-mix. Test that the solution is alkaline (add a little more ammonium hydroxide if necessary, but replace the solution if more than 0.1 ml extra is required), then add approximately 5 µl of the antifoam agent octan-2-ol (**4.20**) and 0.1 ml of ammonium hydroxide/sodium borohydride solution (**4.5**), vortex-mix.

Leave the tubes in a heating block (**5.9**) or water-bath (**5.4**) at 40°C for 30 min. then remove and add 0.2 ml of glacial acetic acid (**4.1**), mix again. Remove 0.5 ml to a 30 ml glass tube; add 0.5 ml of 1-methylimidazole (**4.19**). Add 5 ml of acetic anhydride (**4.2**) and vortex-mix IMMEDIATELY. Leave the tubes for 10 min. for the reaction to proceed (the reaction is exothermic and the tubes will become hot).

Add 0.9 ml of ethanol, absolute, vortex-mix and leave for 5 min. Add 10 ml of water, vortex-mix and leave for 5 min. Add 0.5 ml of bromophenol blue solution (**4.7**). Place the tubes in ice–water and add 5 ml of potassium hydroxide, 7.5 mol/l (**4.21**); a few minutes later add a further 5 ml of potassium hydroxide, 7.5 mol/l, cap the tubes and mix by inversion.

Leave the tubes until the separation into two phases is complete (10 to 15 min.) or centrifuge for a few minutes. Draw the upper phase into the tip of an automatic pipette; if any of the blue phase is included, allow it to separate then run it out of the tip before transferring a portion of the upper phase alone into a small (auto-injector) vial.

7.3 GLC measurement of neutral sugars

Carry out conventional GLC (**5.7**) measurement of the neutral sugars. At the beginning of each batch of analyses, equilibrate with the isothermal elution conditions for at least 1 hr. Carry out several calibration runs to check that the response factors are reproducible. Inject 0.5 to 1 µl of the alditol acetate derivatives prepared in step **7.2.** Use a plunger-in-the-barrel type of needle (e.g. SGE; SR-SS-GT-7T) and pre-pierced septa (e.g. SGE; 7 TCSD P/N 041852).

7.3.1 GLC column and conditions

Injector temperature, 275 °C; detector temperature, 275 °C; carrier gas, helium. Under these conditions, a GLC chromatograph fitted with flame ionization detector and, preferably, auto-injector and computing integrator, using a Supelco SP-2380 wide-bore capillary column (30 m × 0.53 mm id) with a temperature gradient (0.5 min. at 180°C, ramp 5 deg. C/min. to upper temperature of 215°C, upper time 2 min.; helium flow-rate 16 ml/min.) allows accurate determination of the individual sugars in the standard sugar mixture to be obtained with a run-time of about 8 min.

7.3.2 GLC calibration

Start at a flow-rate of 12 ml/min and inject a standard that has been reduced and derivatized according to the procedure. Increase the flow-rate by approx. 2 ml/min. and inject another portion of the standard. Repeat as necessary. Increasing the flow-rate will result in a narrower solvent front, giving greater separation between the front and the sugar peaks. An excessive flow-rate will result in a broadening of the solvent front. The optimum flow-rate is just below the rate at which this broadening of the solvent front occurs, and is usually about 16 ml/min. using the operating conditions described here.

For calibration, use the following ratio for the combination of the standard sugar mixture and internal standard (allose):

Sugar	Actual	NSP recovery	Apparent	Calibration
Rhamnose	520	52	1000	1
Fucose	480	96	500	0.5
Arabinose	4750	95	5000	5
Xylose	4450	89	5000	5
Mannose	2300	92	2500	2.5
Galactose	2820	94	3000	3
Glucose	9400	94	10,000	10
Allose	–	–	–	3

The Actual column shows the amount of each sugar in the stock sugar mixture, and the Apparent column shows the values to be used for calibration. These values take into account the recovery of NSP constituents. The Calibration ratio column gives the ratio of sugars to the internal standard after the addition of allose to the standard sugar mixture in step **7.2.**

7.4 Calculation of neutral sugars as measured by GLC

All calculations can be done automatically with a computing integrator. The amount of each individual sugar (expressed as grams of polysaccharide per 100 g of sample) is calculated as:

$$[(A_t \times W_i \times R_f \times 100)/(A_i \times W_t)] \times 0.89$$

where:

A_t and A_i are the peak areas of the test sample and the internal standard, respectively;

W_i is the weight (in mg; here 15 mg; total hydrolysate 30 ml × 0.5 mg of allose) of the internal standard;

R_f is the response factor for individual sugars obtained from the calibration run with the sugar mixture treated in parallel with the samples;

W_t is the weight (in mg) of the test sample;

0.89 is the scale factor for converting the experimentally determined values for monosaccharides to polysaccharides.

7.5 Uronic acid standard solution

The standard sugar mixture in sulphuric acid, 2 mol/l, prepared in step **7.1** contains, for the purposes of calculation, 0.5 mg of galacturonic acid/ml.

To prepare the uronic acid standard solutions, take 0.5 ml, 2.0 ml and 3.0 ml of the sugar mixture into separate tubes and dilute to 10.0 ml with sulphuric acid, 2 mol/l (**4.27**), to give standards of 25, 100 and 150 µg of galacturonic acid/ml. Only the 100 µg/ml standard is required for routine analysis (it may be kept at 5°C for several weeks).

7.6 Measurement of uronic acids by colorimetry

Place into separate tubes (40-50 ml capacity) 0.3 ml of blank solution (sulphuric acid, 2 mol/l), 0.3 ml of each of the standard solutions and 0.3 ml of the test sample hydrolysates, diluted if necessary (with sulphuric acid, 2 mol/l) to contain not more than 150 µg of uronic acids per ml (e.g. no dilution for flour, 1:2 for bran, 1:5 for most fruits and vegetables). Add 0.3 ml of sodium chloride/boric acid solution (**4.24**) and mix. Add 5 ml of sulphuric acid, concentrated (**4.26**) and vortex-mix immediately. Place the tubes in a heating block or water-bath at 70°C and leave them there for 40 min. Remove the tubes and cool to room temperature in water (the tubes may be kept in the water for up to 1 hr.).

Add 0.2 ml of dimethylphenol solution (**4.8**) and vortex-mix immediately. After 15 min. measure the absorbance at 400 nm and at 450 nm in the spectrophotometer (**5.5**) against the blank solution. The timing for measurement of the absorbance of standards and samples should be identical. In practice, this is achieved by adding the chromogenic reagent at 1 min. intervals. Subtract the reading at 400 nm from that at 450 nm, to correct for interference from hexoses. A straight line passing through the origin should be obtained if the differences for the standards are plotted against concentration.

7.7 Calculation of uronic acids as measured by colorimetry

The amount of uronic acids (expressed as grams of polysaccharide per 100 g of sample) is calculated as:

$$[(Abs_t \times V_t \times D \times C \times 100)/(Abs_S \times W_t)] \times 0.91$$

where:

Abs_t is the difference in absorbance of the test solution;

V_t is the total volume of test solution (here 30 ml);

D is the dilution of the test solution;

C is the concentration of the standard (here 0.1 mg/ml);

Abs_S is the difference in absorbance of the 0.1 mg /ml standard;

W_t is the weight (in mg) of the test sample;

0.91 is the scale factor for converting the experimentally determined values for monosaccharides to polysaccharides.

7.8 Calculation of total, soluble and insoluble NSP

The amount of total, soluble and insoluble NSP, in g/100 g of sample, is calculated as:

Total NSP = Neutral sugars calculated for portion (a) [step **7.4**]

+ Uronic acids calculated for portion (a) [step **7.7**]

Insoluble NSP = Neutral sugars calculated for portion (b) [step **7.4**]

+ Uronic acids calculated for portion (b) [step **7.7**]

Soluble NSP = Total NSP – Insoluble NSP

It is recommended that the results for total, insoluble and soluble NSP are expressed as grams of polysaccharide per 100 g of dry matter.

For a detailed description of the separate measurement of cellulose, see reference **9.7**.

7.9 Breaks in the procedure

The procedure may be halted at any of the following stages.

Ater precipitation, washing and drying the starch-free residue in steps **6.5** and **6.6**. The residue may be stored for long periods.

After the hydrolysis with sulphuric acid, 2 mol/l, in step **6.7**. The hydrolysate may be kept at 4°C for 24 hr.

After acidification of the reduced samples in step **7.2**. The samples may be stored at room temperature for two or three days.

After acetylation and transfer to small vials in step **7.2.** The samples may be kept at 0 to 5°C for 24 hr before analysis by GLC.

The acid hydrolysate in step **6.7.1** may be kept at 5°C for several weeks before the measurement of uronic acids.

7.10 Trouble shooting

See Appendix II.

8. VALIDATION

The procedure, essentially as described in this protocol, has been subjected to an international collaborative trial organised by the UK Ministry of Agriculture, Fisheries and Food as part of its Collaborative Trial Programme. The format and rationale of these trials is described in MAFF Validated Method V0. The results of the collaborative trial are given in reference **9.11** and summarised in Appendix III.

Minor modifications since the collaborative trial have made the technique more rapid and robust (**9.7**). The improved procedure has been trialled by BCR, leading to the issue of five certified reference materials (**9.14**). The procedure is approved by MAFF for food labelling within UK.

A comparison of dietary fibre values obtained by the Englyst procedure (**9.7**) and by the AOAC Prosky procedure (**9.15**) for components of the UK diet is given in reference **9.13**, where the reasons for the differences between the values obtained by these methods are discussed.

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APPENDIX I

DIAGRAM FOR THE ANALYSIS OF DIETARY FIBRE AS NSP

SAMPLE

Add 2 ml DMSO

↓

30 min. at 100 °C

↓

Add 8 ml enzyme solution I

↓

10 min. at 100 °C

↓

Add 0.5 ml enzyme solution II

↓

30 min. at 50 °C; 10 min. at 100 °C

↓

Cool and add 0.15 ml HCl, 5 mol/l
Add 40 ml acidified ethanol

↓

30 min. at 0 °C

↓

Centrifuge, wash with acidified 85% ethanol; wash with ethanol; dry with acetone

↓

Add 5 ml sulphuric acid, 12 mol/l

↓

30 min. at 35 °C

↓

Add 25 ml water

↓

1 hr. at 100 °C

↓

HYDROLYSATE

NEUTRAL SUGARS — GLC

Add to 1 ml; 0.5 ml internal standard,
0.4 ml ammonia solution, 12 mol/l,
5 ml octan-2-ol,
0.1 ml sodium borohydride solution
30 min. at 40 °C

↓

Add 0.2 ml acetic acid

↓

Add to 0.5 ml;
0.5 ml l-methylimidazole,
5 ml acetic anhydride
Leave for 10 min.

↓

Add 0.9 ml ethanol
Leave for 5 min.

↓

Add 10 ml water,
2 × 5 ml KOH, 7.5 mol/l

↓

Use top phase for measurement of neutral sugars

URONIC ACIDS

Add to 0.3 ml;
0.3 ml sodium chloride/ boric acid solution
5 ml sulphuric acid, conc.

↓

40 min. at 70 °C

↓

Cool to room temperature, add 0.2 ml dimethylphenol solution
Leave for 15 min

↓

Read absorbance at 400 nm and 450 nm

APPENDIX II
TROUBLE-SHOOTING GUIDE

Problems associated with preparation of the hydrolysate

Symptom	Likely cause	Prevention/Cure
Good duplicates (within batch) + good replicates (between batch) but values lower or higher than expected for material of known composition	Systematic error e.g. weighing, pipetting, determination of moisture content	Calibrate balance using 1 g weight. Use positive displacement pipettes, all calibrated by weighing replicates of water. Check moisture content of reference samples
Values higher than expected and variable for duplicates	Incomplete removal of starch leading to high glucose values	Vortex-mix vigorously at all the times indicated. Check the pH of the buffers used and temperature of water-baths
Values lower than expected and variable for duplicates.	Incomplete depolymerisation during acid hydrolysis	Prevent aggregation of the sample during the drying step by regular, vigorous vortex-mixing.

Problems associated with the GLC end-point

Symptom	Likely cause	Prevention/Cure
Variation between replicate analyses	Incorrect calibration of the GLC instrumentation	Check the calibration of all instrumentation. For the GLC perform analysis only after the response factors for the standard sugar mixture are consistent and similar to those obtained previously. Keep a record of response factors
Extra peaks on the chromatogram	Incomplete reduction/storage of samples	Ensure alkaline pH before adding $NaBH_4$. Replace old $NaBH_4$. Use freshly prepared derivatives
Little or no upper phase for GLC	Very high protein in sample	Use 1 ml ethanol instead of 0.9 ml and centrifuge the derivative
Broad solvent front	Column set up incorrectly	Check setting-up procedure

APPENDIX III

SUMMARY OF RESULTS FOR INDIVIDUAL SAMPLES

Sample	Fibre Type	Mean	r	R
Coconut	Soluble	1.21	1.46	2.54
	Insoluble	11.12	1.61	3.10
	Total	12.31	2.61	3.03
Porridge	Soluble	3.68	1.35	1.94
	Insoluble	3.42	0.77	1.46
	Total	7.22	1.13	2.27
Baked Beans	Soluble	7.06	1.27	1.95
	Insoluble	5.56	0.86	1.80
	Total	12.62	1.07	3.06
Cabbage	Soluble	12.15	4.24	6.61
	Insoluble	13.04	1.80	4.93
	Total	25.25	3.17	8.54
Mashed Potato	Soluble	4.03	0.84	0.97
	Insoluble	2.83	0.84	1.29
	Total	6.87	0.67	1.44
Banana	Soluble	2.88	0.80	1.57
	Insoluble	1.87	0.65	0.97
	Total	4.62	0.76	1.48
Apple	Soluble	5.28	1.21	2.21
	Insoluble	6.45	1.10	2.45
	Total	11.73	1.06	3.79
Bread	Soluble	2.82	0.61	1.61
	Insoluble	2.77	0.48	0.96
	Total	5.61	0.66	1.96
White Bread	Soluble	1.83	1.50	1.50
	Insoluble	1.61	0.74	0.91
	Total	3.43	0.99	1.33
Corn Flakes	Soluble	0.46	0.47	0.66
	Insoluble	1.04	0.69	1.69
	Total	1.48	0.73	1.54
Wholemeal Bread	Soluble	2.50	1.20	1.20
	Insoluble	7.01	1.05	2.10
	Total	9.30	1.13	1.88
1:1 Bread Mix	Soluble	2.16	1.33	1.39
	Insoluble	4.41	0.79	1.37
	Total	6.56	0.92	1.67

J. Assoc. Publ. Analysts, 1997, 33, 145-159

MAFF VALIDATED METHODS FOR THE ANALYSIS OF FOODSTUFFS

DIETARY FIBRE:ENGLYST PROCEDURE FOR DETERMINATION OF DIETARY FIBRE AS NON-STARCH POLYSACCHARIDES:

MEASUREMENT OF CONSTITUENT SUGARS BY COLORIMETRY

V40

Correspondence on this method may be sent to Hans Englyst, Dunn Clinical Nutrition Centre, Hills Road, Cambridge, CB2 2DH

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 7UQ

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, with respect to the sample preparation.

1. SCOPE AND FIELD OF APPLICATION

The Englyst procedure determines total, soluble and insoluble non-starch polysaccharides (NSP) in plant foods and food products. The cell-wall material naturally present in plants is the common characteristic of the foods that comprise a high-fibre diet and approximately 90% of this material is NSP. The specific measurement of the NSP content of unfortified plant foods provides a good marker for endogenous plant cell-wall material and, therefore, for a high-fibre diet (i.e. a diet rich in plant cell-wall material, minerals, vitamins and antioxidants but low in fat and sodium).

The EC Scientific Committee for Food have been considering the definition of dietary fibre for food labelling and have reported (**9.1**):

"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."

NSP values will aid the consumer in choosing the type of naturally high-fibre diet for which health benefits have been shown, and which is recommended in national dietary guidelines.

The procedure is widely used in research, and it is one of the methods used for food labelling for dietary fibre within the EC.

NSP values are used in European nutritional databases and are reported as dietary fibre in food tables (e.g. **9.2 - 9.4**).

2. DEFINITION

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

3. PRINCIPLE

The Englyst procedure (**9.5 - 9.8**) measures non-starch polysaccharides (NSP), using enzymic-chemical methods, and has evolved from the principles laid down by McCance and Widdowson, and later by Southgate (**9.9**). In the procedure, starch is dispersed and then hydrolysed enzymically. The NSP are precipitated with alcohol and hydrolysed by sulphuric acid, releasing neutral sugars and uronic acids, which are measured by colorimetry (**9.6, 9.7**). Values for total, soluble and insoluble NSP as the sum of individual neutral sugars and uronic acids may be obtained within an 8 hr. working day. The assay is suitable for routine analysis and the end-point may be automated (**9.5**).

(When details of the component sugars are required, the use of a GLC end-point for measurement of individual neutral sugars allows NSP values to be obtained within 1.5 working days (**9.7, 9.12**). Agreement is good between the GLC and colorimetric assay).

The Englyst procedure for the measurement of NSP by colorimetry is summarised in Appendix I.

4. REAGENTS

All the reagents marked with an asterisk (*), together with four reference samples, are available in Kit form from the MRC Dunn Clinical Nutrition Centre.

High-purity reagents and distilled water, or water of an equivalent purity, should be used throughout the method.

4.1 **Acetic acid**, glacial

4.2 **Acetone**

4.3 **Benzoic acid**, 50% saturated: prepare by diluting saturated benzoic acid 1:1 v/v with water.

4.4 **Colorimetry stock sugar mixture**. Make the stock sugar solution by weighing (all sugars dried to constant weight under reduced pressure with phosphorus pentoxide) to the nearest 1 mg; 10.185 g of arabinose, 5.145 g of glucose and 2.16 g (2.36 g of the monohydrate) of galacturonic acid. Place them in a 1 litre calibrated flask and dilute to volume with 50% saturated benzoic acid (**4.3**). Store the mixture at 4°C.*

4.5 **3,3-Dimethylglutaric acid stock solution**, 0.5 mol/l. The purity of 3,3-dimethylglutarate (DMG) varies between Lot numbers. The following procedure should be used for each new Lot number in the preparation of the DMG solution. Weigh 8 g of DMG (dried under reduced pressure with phosphorus pentoxide) to the nearest 1 mg, dissolve in 98.5 g of NaOH, 5 mol/l (**4.20**) and add 1 ml of water. Dilute a portion of the stock solution 1:1 v/v with sulphuric acid, 2 mol/l (**4.23**); the pH must be 3.75 (± 0.15) at room temperature. If the pH is greater than 3.9, add 1 ml of water to the stock solution, mix and remove a portion to check the pH again. If the pH is less than 3.6, add 1 ml of NaOH, 5 mol/l (**4.20**) to the DMG stock solution, mix and remove a portion to check the pH again. Repeat as necessary.

4.6 **3,5-Dimethylphenol**, dissolve 0.1 g of 3,5-dimethylphenol ($(CH_3)_2C_6H_3OH$, Aldrich cat. no.14,413-4) in 100 ml of glacial acetic acid (**4.1**). [See **7.3**]

4.7 **Dimethyl sulphoxide**

4.8 **Dinitrosalicylate**, dissolve 10 g of 3,5-dinitrosalicylic acid ($C_7H_4N_2O_7$; Sigma cat. no. D 0550) and 300 g of sodium/potassium tartrate ($C_4H_4KNaO_6.4H_2O$; Sigma cat. no. S2377) in approx. 300 ml of water plus 400 ml of NaOH, 1 mol/l (**4.20**). Dissolve by stirring (overnight) and make to a final volume of 1 litre with water. Sparge for 10 min. with helium or nitrogen, or degas using an ultrasonic bath. Store in well-capped

opaque bottles at 0-4 °C. Before use, bring to room temperature and mix well to ensure that any precipitate formed during storage is dissolved. The reagent is stable at room temperature for several months and at 0-4 °C for at least 1 year.*

4.9 **Enzyme solution I**, take 2.5 ml of heat-stable amylase (EC. 3.2.1.1; Termamyl 300 KNU/g Novo Nordisk*) and make to 200 ml with the pre-equilibrated sodium acetate buffer (**4.18**), mix, and keep it in a 50°C water-bath (**5.4**). Prepare the solution immediately before use.

4.10 **Enzyme solution II**, take 1.2 g of pancreatin (Pancrex V powder from Paynes & Byrne Ltd, Greenford, Middlesex, UK or Sigma, cat. no. P1500*) into a 50 ml tube, add 12 ml of water, vortex-mix initially and then mix for 10 min. with a magnetic stirrer. Vortex-mix again, then centrifuge for 10 min. Take 10 ml of the (cloudy) supernatant, add 2.5 ml of pullulanase (EC 3.2.1.41; 200 PNU/g Novo Nordisk*) and vortex-mix. Prepare the solution immediately before use and keep it at room temperature.

4.11 **Ethanol**, absolute.

4.12 **Ethanol**, absolute, acidified. Add 1 ml of hydrochloric acid, 5 mol/l, per litre of ethanol (**4.11**).

4.13 **Ethanol**, 85% v/v, acidified. Add 150 ml of water to 1 ml of hydrochloric acid, 5 mol/l (**4.15**), and make to 1 litre with ethanol (**4.11**).

4.14 **Glass balls**, 2.5–3.5 mm diameter (Merck cat. no. 33212).

4.15 **Hydrochloric acid,** 5 mol/l.

4.16 **Pectinase** (EC 3.2.1.15 Novo Nordisk*), add 9 volumes of water to 1 volume of pectinase solution.

4.17 **Sand, acid-washed**, 50–100 mesh (Merck cat. no. 33094).

4.18 **Sodium acetate buffer**, 0.1 mol/l, pH 5.2: adjust sodium acetate, 0.1 mol/l (**4.18.1**), to pH 5.2 with acetic acid, 0.1 mol/l (**4.18.2**). To stabilise and activate enzymes, add 4 ml of calcium chloride, 1 mol/l (**4.18.3**), to 1 litre of buffer.

4.18.1 **Sodium acetate**, 0.1 mol/l: dissolve 13.6 g of sodium acetate trihydrate, $CH_3COONa.3H_2O$, and make to a final volume of 1 litre with water. Stable for several weeks at 0–4 °C.

4.18.2 **Acetic acid**, 0.1 mol/l.

4.18.3 **Calcium chloride**, 1 mol/l.

4.19 **Sodium chloride/boric acid**, dissolve 2 g of sodium chloride, NaCl, and 3 g of boric acid, H_3BO_3, in 100 ml of water. Stable for several months at room temperature. [See **7.3**]

4.20 **Sodium hydroxide**, 1.0 mol/l, 3.0 mol/l and 5 mol/l. Prepare from commercially available standard solutions (e.g. ConvoL®, Merck product no. 18023)Do not use pellets.

4.21 **Sodium phosphate buffer**, 0.2 mol/l, pH 7: adjust Na_2HPO_4, 0.2 mol/l (**4.21.1**), to pH 7 with NaH_2PO_4, 0.2 mol/l (**4.21.2**). Prepare buffer on day of use.

4.21.1 **Disodium hydrogen phosphate**, 0.2 mol/l. Stable for several months at room temperature.

4.21.2 **Sodium dihydrogen phosphate**, 0.2 mol/l. Stable for several months at room temperature.

4.22 **Sulphuric acid**, concentrated.

4.23 **Sulphuric acid**, 2 mol/l. Add 5 ml of sulphuric acid, 12 mol/l (**4.24**) to 25 ml of water. Cool to room temperature before use.

4.24 **Sulphuric acid**, 12 mol/l. This may be obtained as sulphuric acid, 72% w/w (Merck cat. no. 19321 6Y).

4.25 **Sulphuric acid**, 2.4 mol/l. Add 5 ml of sulphuric acid, 12 mol/l (**4.24**), to 20 ml of water. Cool to room temperature before use.

5. APPARATUS

5.1 **Balance**, accurate to 0.1 mg.

5.2 **Centrifuge**, capable of exerting 1500 *g*.

5.3 **Centrifuge tubes**, glass centrifuge tubes of 50-60 ml capacity (e.g. Anachem cat. no. 45212-50), fitted with Teflon-lined screw tops; 24 tubes are suitable for a batch. Make a mark at 50 ml.

5.4 **Water-baths**, one capable of maintaining 100°C; one capable of maintaining temperatures in the range 35°C to 70°C. Each bath should be of such a capacity that there is no significant change in temperature when a rack containing all the tubes (**5.3**) is placed in it.

5.5 **Spectrophotometer**, capable of measurement at 530 nm.

5.6 **Vortex-mixer**

5.7 **Fan oven,** capable of maintaining 80°C.

5.8 **Heating block**, capable of maintaining 40°C and 70°C.

6. PROCEDURE

6.1 Pre-treatment of samples

All samples should be finely divided so that representative sub-samples may be taken. Foods with a low water content (<10%) may be milled, and foods with a higher water content may be homogenised wet or milled after freeze-drying.

6.2 Test samples and sample weights

6.2.1 Test samples

Two portions, (a) and (b), of each test sample are required to obtain separate values for total, insoluble and soluble NSP. Portion (a) is used to measure total NSP; portion (b) is used to measure insoluble NSP. Soluble NSP is determined as the difference. The two portions are treated identically throughout the procedure, except for their separate treatment in steps **6.5** and **6.6**.

6.2.2 Sample weight

Weigh (**5.1**), to the nearest 0.1 mg, between 50 and 1000 mg depending on the water and NSP content of the sample (to give not more than 300 mg of dry matter; e.g. 300 mg is adequate for most dried foods but use 100 mg for bran and purified fibre preparations) into 50-60 ml screw-top glass tubes (**5.3**). Add 300 (±20) mg of acid-washed sand (**4.17**) and approximately 15 glass balls (**4.14**) to each tube. If the sample is dry (90 to 100% dry matter) and contains less than 10% fat, proceed to step **6.4**; otherwise, go to step **6.3**. (It is recommended that all analyses are carried out in duplicate.)

6.3 Fat extraction/drying

Add 40 ml of acetone (**4.2**), cap the tubes and mix several times over a 30 min. period. Centrifuge (**5.2**) at 1000 *g* for 10 min. to obtain a clear supernatant liquid and remove by aspiration as much of the supernatant liquid as possible without disturbing the residue. Discard the supernatant. Vortex-mix vigorously to ensure that the residue is dispersed thinly around the bottom 5 cm of the tube.

Place the tubes in a pan of water at 75°C in a fume-cupboard. Remove the tubes singly and vortex-mix vigorously at frequent intervals until the tubes and residues are dry. (Note: an evaporator may be used, see **6.5.4**.)

6.4 Dispersion and enzymic hydrolysis

6.4.1 Treatment with dimethyl sulphoxide (DMSO)

Pre-equilibrate sufficient acetate buffer (**4.18**) at 50°C (8 ml required per sample).

Add 2 ml of DMSO (**4.7**) to the dry sample, cap the tube, and immediately mix the contents using a vortex-mixer (**5.6**). It is essential that all the sample is wetted and no material is encapsulated or adhering firmly to the tube wall before proceeding. When DMSO has been added and mixed in all the tubes, vortex-mix a further three or four times during a 5 min. period.

Vortex-mix and immediately place two of the tubes into a boiling water-bath (**5.4**). Remove after 20 seconds, vortex-mix and replace into the boiling water-bath. Repeat this for subsequent pairs of tubes until all the tubes are in the bath; leave them there for 30 min. from that time. During this period, prepare enzyme solutions I (**4.9**) and II (**4.10**). The volumes given are suitable for 24 tubes.

6.4.2 Treatment with enzymes

Remove one tube at a time, vortex-mix, uncap and immediately add, by positive displacement, 8 ml of enzyme solution I (**4.9**), cap the tube, vortex-mix thoroughly, ensuring that no material adheres firmly to the tube wall, and replace it in the boiling water-bath. Leave the tubes there for 10 min., timed from the last addition of enzyme.

Transfer the rack of tubes to the 50°C water-bath (**5.4**). After 3 min., add 0.5 ml of enzyme solution II (**4.10**) to each tube and mix the contents thoroughly to aid distribution of the enzyme throughout the sample. Replace the tubes in the 50°C water-bath and leave them there for 30 min. Mix the contents of each tube continuously or after 10 min., 20 min. and 30 min. Transfer the rack of tubes to the boiling water-bath and leave them there for 10 min.

6.5 Precipitation and washing of the residue for measurement of total NSP: test sample portion (a) only

6.5.1 Cool the sample by placing in ice–water. Add 0.15 ml of hydrochloric acid, 5 mol/l (**4.15**), and vortex-mix thoroughly two or three times during a 5 min. period, with tubes being replaced in the ice–water. Add 40 ml of acidified ethanol, absolute (**4.12**), to give a final concentration of 80% v/v. Mix well by repeated inversion, then leave in ice–water for 30 min.

Centrifuge (**5.2**) at 1500 *g* for 10 min. to obtain a clear supernatant liquid. Remove by decanting or by aspiration as much of the supernatant liquid as possible, without disturbing the residue, and discard the liquid.

6.5.2 Add approximately 10 ml of acidified ethanol, 85% (**4.13**), to the residue and vortex-mix. Make to 50 ml with acidified ethanol, 85% (**4.13**), cap the tube and mix thoroughly by repeated inversion. Centrifuge and remove the supernatant liquid as above. Repeat this stage using 50 ml of ethanol, absolute (**4.11**).

6.5.3 Add 30 ml of acetone (**4.2**) to the residue and vortex-mix thoroughly to form a suspension. Centrifuge and remove the supernatant liquid as in step **6.5.1**.

6.5.4 Vortex-mix vigorously to ensure that the residue is dispersed thinly around the bottom 5 cm of the tube. Place the rack of tubes in a pan of water at 70 to 75°C in a fume-cupboard. Remove the tubes singly and vortex-mix vigorously at frequent intervals, to ensure that the residue in each tube is finely divided, until the tube and residue appear dry. Place the rack of tubes in a fan oven (**5.7**) at 80°C for 10 min. to remove any traces of acetone. It is essential that the residues and tubes are completely free of acetone. (Instead of using the fume-cupboard/drying oven, it is convenient to dry the residue in an evaporator (e.g. TurboVap, Zymark Ltd, Cheshire, UK). After 1 min. mix again and replace the tubes in the apparatus. Repeat until the residue is free of lumps and the tubes and residues are free of acetone.)

6.6 Extraction and washing of the residue for measurement of insoluble NSP: test sample portion (b) only

6.6.1 After the treatment with enzymes in step 6.4, add 40 ml of sodium phosphate buffer (**4.21**). Place the capped tubes in the boiling water-bath and leave them there for 30 min. Mix continuously or a minimum of three times during this period. Remove the tubes and equilibrate to room temperature in water. Centrifuge and remove the supernatant liquid as in step **6.5.1**.

6.6.2 Add approximately 10 ml of water and vortex-mix. Make to 50 ml with water and mix well by repeated inversion. Centrifuge (**5.2**) and remove the supernatant liquid as above. Repeat this stage using 50 ml of ethanol, absolute (**4.11**).

6.6.3 Proceed as described for step **6.5.3** and step **6.5.4**.

6.7 Acid hydrolysis of the residue from steps **6.5** and **6.6**

6.7.1 Add 5 ml of sulphuric acid, 12 mol/l (**4.24**), to one tube and immediately vortex-mix vigorously; ensure that all the material is wetted. Repeat this for each tube in turn. Once the acid has been added to all the tubes, vortex-mix again and place the tubes into a water-bath at 35°C (**5.4**). Leave the tubes for 30 min. with vigorous vortex-mixing after 5, 10 and 20 min. to disperse cellulose. To each tube in turn, add 25 ml of water rapidly from a positive displacement device and vortex-mix. Place all the tubes into a boiling water-bath (**5.4**) and leave them there for 1 hr., timed from when boiling recommences; mix after 10 min. Remove all the tubes together and cool rapidly in water.

7. MEASUREMENT OF CONSTITUENT SUGARS BY COLORIMETRY

7.1 Preparation of standard sugar solutions for colorimetry

Take 0.5 ml of the stock sugar mixture (**4.4**) into a glass tube, add 2.5 ml of sulphuric acid, 2.4 mol/l (**4.25**), and mix to give 3 ml of 3 mg sugars/ml standard solution in sulphuric acid, 2 mol/l. The chromogenic reaction is linear up to 3 mg sugars /ml. The absorbance of the samples should not exceed that of the standard.

7.2 Measurement of total reducing sugars

Place into separate tubes 0.5 ml of the standard solution, 0.5 ml of the hydrolysate from step **6.7**, and into each of a further two tubes (blanks 1 and 2), 0.5 ml of sulphuric acid, 2 mol/l (**4.23**). Add 0.5 ml of dimethylglutarate solution (**4.5**) and vortex-mix. Check the pH of one drop of blank 1; it should be between 3.5 and 4. Add 0.1 ml of diluted pectinase solution (**4.16**), vortex-mix and place all the tubes into a water-bath at 50°C; leave them there for 20 min. Cool the tubes to room temperature and add 0.1 ml of sodium hydroxide, 3 mol/l (**4.20**), vortex-mix and leave for 5 min. Check that the pH of blank 1 is between 6 and 8. Add 1 ml of dinitrosalicylate reagent (**4.8**) to each tube and vortex-mix. Place all the tubes together into a briskly boiling water-bath and leave them there for 5 min. Remove the rack of tubes and cool them to room temperature in water. Add 10 ml of water and mix by inversion (do not use the vortex-mixer at this stage).

Measure the absorbance in the spectrophotometer (**5.5**) at 530 nm against blank 2.

[Sample blanks may be prepared by diluting the hydrolysate as described above, replacing the dinitrosalicylate reagent with water and reading the absorbance against water. Alternatively, the absorbance of the undiluted

hydrolysate can be measured against water and the value obtained divided by 24.4, the dilution of the hydrolysate after addition of the dinitrosalicylate reagent and water. The absorbance of the test sample is then calculated by subtracting this value. When the hydrolysate is colourless and the NSP content is more than 5%, the sample blank is not required.]

7.3 Calculation of total, soluble and insoluble NSP as measured by colorimetry

The amount of total NSP (portion (a)) and of insoluble NSP (portion (b)) in g/100 g of sample, is calculated as:

$$[(Abs_t \times V_t \times D \times F \times C \times 100)/(Abs_S \times W_t)] \times 0.89$$

where :

Abs_t is the absorbance of the test solution (minus the absorbance of the hydrolysate blank if measured);

V_t is the total volume of the test solution (here, 30 ml);

D is the dilution of the test solution; (D =1 if no dilution in step 7.2);

F is the factor correcting the difference between the composition of monosaccharides in the sugar mixture and that in NSP of various types of plant foods.

For the calculation of total NSP: cereals, F = 0.95; fruit and non-starchy vegetables, F = 1.05; starchy vegetables, oats and unknown samples, F = 1.

For the calculation of insoluble NSP: F = 1 except for cereals, where F = 0.95;

C is the concentration (in mg sugars/ml) of the standard used (here, C = 3);

Abs_S is the absorbance of the 3 mg/ml standard;

W_t is the weight (in mg) of sample taken for analysis;

0.89 is the factor for converting the experimentally determined monosaccharides to polysaccharides.

The amount of soluble NSP is calculated as the difference between total and insoluble NSP. It is recommended that values are expressed as grams of polysaccharide per 100 g of dry matter.

(For a detailed description of the separate measurement of uronic acids (requires reagents **4.6, 4.19** and **4.22**) and cellulose, see reference **9.7**.)

7.4 Breaks in the procedure

The analysis may be completed in one day if desired. However, the procedure may be halted at either of the following stages.

After precipitation, washing and drying the starch-free residue in steps **6.5** and 6.6. The residue may be stored for long periods.

After the hydrolysis with sulphuric acid, 2 mol/l, in step **6.7**. The hydrolysate may be kept at 4°C for 24 hr.

7.5 Trouble shooting

See Appendix II.

8. VALIDATION

The procedure, essentially as described in this protocol, has been subjected to an international collaborative trial organised by the UK Ministry of Agriculture, Fisheries and Food as part of its Collaborative Trial Programme. The format and rationale of these trials is described in MAFF Validated Method V0. The results of the collaborative trial are given in reference **9.11** and summarised in Appendix III.

Minor modifications to the Englyst colorimetric procedure since the collaborative trial have made the technique more rapid and robust (**9.7**). The improved procedure has been trialled by BCR, leading to the issue of five certified reference materials (**9.14**). The procedure is approved by MAFF for food labelling within UK.

A comparison of dietary fibre values obtained by the Englyst procedure (**9.7**) and by the AOAC Prosky procedure (**9.15**) for components of the UK diet is given in reference **9.13**. That paper discusses the reasons for the differences between the values obtained by these methods.

9. REFERENCES

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APPENDIX I

DIAGRAM FOR THE ANALYSIS OF DIETARY FIBRE

SAMPLE

↓

Add 2 ml DMSO

↓

30 min. at 100 °C

↓

Add 8 ml enzyme solution I

↓

10 min. at 100 °C

↓

Add 0.5 ml enzyme solution II

↓

30 min. at 50 °C; 10 min. at 100 °C

↓

Cool and add 0.15 ml HCl, 5 mol/l

↓

Add 40 ml acidified ethanol

↓

30 min. at 0 °C

↓

Centrifuge,
wash with acidified 85% ethanol;
wash with ethanol; dry with acetone

↓

Add 5 ml sulphuric acid, 12 mol/l

↓

30 min. at 35 °C

↓

Add 25 ml water

↓

1 hr. at 100 °C

↓

HYDROLYSATE

↓

Add 0.1 ml NaOH, 3 mol/l

↓

Add to 0.5 ml;
0.5 ml dimethylglutaric acid
0.1 ml dilute pectinase

↓

20 min. at 50°C

↓

Leave 5 min. at room temperature

↓

Add 1 ml colour reagent

↓

5 min. at 100 °C

↓

Add 10 ml water

↓

Read absorbance at 530 nm

APPENDIX II
TROUBLE-SHOOTING GUIDE

Problems associated with preparation of the hydrolysate

Symptom	Likely cause	Prevention/cure
Good duplicates (within batch) + good replicates (between batch) but values lower or higher than expected for material of known composition	Systematic error e.g. weighing, pipetting, determining moisture content	Calibrate balance using 1 g weight. Use positive displacement pipettes, all calibrated by weighing replicates of water. Check moisture content of the reference samples
Values higher than expected and variable for duplicates	Incomplete removal of starch leading to high glucose values	Vortex-mix vigorously at all the times indicated. Check the pH of the buffers used and temperature of water-baths
Values lower than expected and variable for duplicates	Incomplete depolymerisation during acid hydrolysis	Prevent aggregation of the sample during the drying step by regular, vigorous vortex-mixing.

Problems associated with the colorimetric end-point

Symptom	Likely cause	Prevention/cure
Values higher than expected and variable	Incomplete removal of acetone	Ensure there is no trace of acetone. Dry in oven longer
No colour produced for standards and/or samples	Error in preparation of sulphuric acid, sodium hydroxide or dimethylglutaric acid solution	Use commercially prepared solutions where indicated. Check reagents, including dimethylglutarate solution
Values for fruit and vegetables lower than expected	Incomplete hydrolysis of pectin	Ensure correct pH and temperature during hydrolysis with pectinase. Check reagents as above

APPENDIX III

SUMMARY OF RESULTS FOR INDIVIDUAL SAMPLES

Sample	NSP Type	Mean	r	R
Coconut	Soluble	1.39	1.09	2.82
	Insoluble	11.71	0.95	4.62
	Total	13.06	0.91	4.83
Porridge	Soluble	4.46	1.56	2.38
	Insoluble	4.07	0.65	2.67
	Total	8.69	0.72	2.21
Baked Beans	Soluble	7.29	1.88	3.00
	Insoluble	6.16	0.89	2.65
	Total	13.13	4.52	4.52
Cabbage	Soluble	9.93	3.07	5.05
	Insoluble	13.61	1.16	4.59
	Total	23.80	2.89	5.92
Mashed Potato	Soluble	3.85	0.75	0.97
	Insoluble	2.94	0.79	1.80
	Total	7.12	0.89	2.81
Banana	Soluble	2.73	1.60	1.66
	Insoluble	2.46	0.93	1.79
	Total	5.40	1.02	2.61
Apple	Soluble	4.13	2.16	3.50
	Insoluble	6.93	0.89	3.83
	Total	10.78	1.55	6.23
Bread	Soluble	3.39	1.28	1.43
	Insoluble	3.18	0.97	1.23
	Total	6.57	0.83	1.98
White Bread	Soluble	2.11	1.78	1.92
	Insoluble	2.00	1.05	1.35
	Total	4.16	1.62	2.42
Corn Flakes	Soluble	0.68	1.57	1.57
	Insoluble	1.52	0.80	2.71
	Total	2.35	1.05	3.74
Wholemeal Bread	Soluble	2.88	1.45	1.64
	Insoluble	7.71	1.74	3.43
	Total	10.54	1.62	3.08
1:1 Bread Mix	Soluble	2.65	1.18	1.42
	Insoluble	4.87	0.97	2.42
	Total	7.42	0.93	2.44