J.Assoc.Publ.Analysts. 1992, 28, 1-10

# Determination of the Levels of Aflatoxin in Peanut Butter using the Aflaprep Immunoaffinity Column Clean-Up Procedure: Collaborative Trial

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**Summary**:

*Twenty one United Kingdom laboratories participated in a collaborative trial to evaluate the Aflaprep immunoaffinity column for the determination of aflatoxins in peanut butter. Each participant received ten randomly numbered samples of roasted peanut butter which were prepared as five sets of blind duplicates. One set of samples was blank, uncontaminated peanut butter. The other four sets of samples contained different concentrations of total aflatoxin - ca 8; 12; 35; 50µg/kg. These latter samples were prepared from naturally contaminated peanuts, the major contamination being from aflatoxin $G_1$. Participating laboratories were instructed to follow a set procedure with regard to the use of the immunoaffinity column for extraction and were to quantitate total aflatoxin levels by HPLC with post-column derivitisation with iodine. The relative standard deviations for reproducibility ($RSD_R$) for the total aflatoxin levels, from the twenty one laboratories, for the four concentrations of contaminated peanut butter, were respectively; 54% (on a mean of 7.0µg/kg); 30% (on a mean of 11.3µg/kg); 35% (on a mean of 33.2µg/kg); and 36% (on a mean of 47.7µg/kg).*

## Introduction:

The four aflatoxins $B_1$, $B_2$, $G_1$, and $G_2$ are toxic metabolites of the fungi Aspergillus flavus and A. parasiticus[1,2]. They are potent liver toxins showing carcinogenic properties, with aflatoxin $B_1$ being the most toxic compound. They can contaminate some cereal crops and ground nuts and their products.

One of the problems concerning the determination of aflatoxins in foodstuffs has been the sample extraction procedure. The immunochemical analytical methods which have been devised simplify and shorten the time required for the clean-up of extracts of aflatoxin from foodstuffs[3,4]. The immunoaffinity column contains a gelatinous suspension of monoclonal antibodies covalently attached to a solid support. Any aflatoxin present in the sample is retained by the antibody within the gel suspension. The column is washed to remove any extraneous non-specific material. The eluate is collected by elution of a suitable organic solvent for analysis by either high performance liquid chromatography (HPLC) or thin layer chromatography (TLC).

Two previous collaborative trials[5,6] evaluated an immunoaffinity column clean-up procedure to determine the aflatoxin levels in peanut butter. Participants in these trials were expert laboratories routinely carrying out aflatoxin analyses. The specific column used was the Total Easi-Extract column from Biocode, UK. In the first trial[5], which involved 10 UK laboratories, each

 

laboratory had to follow a protocol for the use of the column but could choose their own end detection method. In that trial the two levels of aflatoxin $B_1$ contamination were 12 and 35µg/kg respectively. Relative standard deviations for reproducibility[7] ($RSD_R$) for the two levels were 46% and 36%. The second collaborative trial[6] involved 13 laboratories from 7 different countries and analysed total aflatoxin concentrations present. Another difference between this and the first trial was that the instrumental method used was strictly specified namely HPLC with post column derivitisation with iodine. The mean levels of total aflatoxin found were 4, 15, and 35µg/kg with $RSD_R$ values between 33 and 45%. It was concluded that more development was needed before this particular method could be submitted for adoption as an AOAC approved method because the $RSD_R$ values were too high. This report describes a study of twenty one UK laboratories (mostly Public Analysts) using the May & Baker Aflaprep immunoaffinity column for the preparation of peanut butter extracts for aflatoxin analysis. The end-point determination was by HPLC, but the method to be used (see Appendix I, Section 5.8) was not as closely defined as in the second Biocode trial.

The trial was carried out on samples of peanut butter which contained concentrations of total aflatoxins close to 10µg/kg and at levels significantly above that value. 10µg/kg is the most common statutory limit proposed or introduced by regulatory authorities for aflatoxin contamination of nuts and nut products[8].

**Organisation of the Trial:**

The trial was organised by the Food Science Laboratory, MAFF, in conjunction with BFMIRA who supplied the samples and May & Baker Diagnostic Limited who provided immunoaffinity columns and the protocol for the extraction and HPLC determination.

**Participants:**

Nineteen public analysts, one food manufacturer's laboratory and the Laboratory of Government Chemist participated in the trial.

**Method, Reagents, Apparatus and Procedure Used:**

The method used in this trial is given in Appendix I. It requires the quantitative determination by HPLC of the individual aflatoxins present in peanut butter after using the Aflaprep Immunoaffinity Chromatography for sample clean-up.

**Sample Preparation:**

The samples were prepared by BFMIRA U.K. who were asked to prepare a "blank" and four contaminated butters at levels between 5 - 60µg/kg total aflatoxins. The contaminated butters were prepared by blending high level contaminated butter with uncontaminated butter. The highly contaminated butter was produced from naturally contaminated peanuts. Homogeneity data for each of the samples were considered to be satisfactory (Coefficient of variation for total aflatoxins $\leq$ 8%, n=10 for each sample).

For the four samples the percentage of each aflatoxin present (as a function of the total) was the same. About 70% of the total aflatoxin contamination was caused by $G_1$, about 13% of the aflatoxin contamination was caused by $B_1$, with $G_2$ and $B_2$ being responsible for 5% and 12% contamination respectively. The distribution of aflatoxins in the trial samples is unusual even though prepared as

a result of natural contamination. Normally $B_1$ would be the predominant aflatoxin rather than $G_1$. Nevertheless it was concluded that using these samples would provide an additional evaluation of the method. Each participant was given 10 samples (5 pairs, blind duplicates) each coded with an individual 3 figure number.

**Results:**

Participants were asked to report their results for all four levels of aflatoxins obtained, uncorrected for recovery, as this is the preferred approach in submitting data for collaborative trials[7]. The results received from each participant are shown in tables I - V. Each table gives the concentration of aflatoxins $B_1$ and $G_1$ present in each of the blind duplicates. Each table also shows the total aflatoxin concentration ($B_1 + B_2 + G_1 + G_2$) present in each sample. The individual values of $B_2$ and $G_2$ were very small in comparison with the individual values of $B_1$ and $G_1$ (being of the order of about 10%) and so were only considered as part of the total aflatoxin concentration.

**Statistical Analysis of the Results:**

The results obtained were statistically analysed as outlined in the AOAC/ISO/IUPAC harmonised protocol[7] for the analysis of collaborative trial data. For the detection of the outliers two statistical tests are used. Cochran's maximum variance test ($P<0.05$) was used to detect the presence of laboratories with an exceptionally high value for the range. Grubbs test ($P<0.05$) was used to determine whether the extreme values (highest and lowest) for the laboratory means are sufficiently different to warrant their removal from the data set. Outlying results are marked in the tables of results.

For tables I - V, mean concentrations for the samples containing different levels of aflatoxin were calculated on results remaining after removal of outliers. The repeatability (r), reproducibility (R), their standard deviations ($SD_r$, $SD_R$), and the repeatability and reproducibility relative standard deviation ($RSD_r$, $RSD_R$) were also calculated for the naturally contaminated peanut butters; these values are given in tables II - V.

**Discussion:**

The peanut butters analysed consisted of a blank and four contaminated samples. The level of aflatoxin (consisting entirely of aflatoxin $B_1$) in the blank was very low (total 0.19 μg/kg) with many of the results being reported as below the participants' limits of detection. All these results have been noted in table I as 0.0 and because of the large number of such results it was decided only to obtain a mean value for these data, after removal of outliers, as any other more complex statistical evaluations would not produce meaningful results. For the other four samples, where a more detailed statistical evaluation of the results was carried out, mean levels for total aflatoxin were found to be 7.0, 11.3, 33.2 and 47.7 μg/kg. The mean levels of the first two of these samples straddle the common 10 μg/kg statutory limit[8]. $RSD_R$ values were 54% and 30% respectively. This latter figure is probably not significantly better than the $RSD_R$ values for the two high level peanut butters which were 35 and 36%. $RSD_R$ of the assay would not be expected to be greater at 11 μg/kg than at 48 μg/kg.

In general a collaborative trial is aimed at evaluating the analytical procedure without specifying or endorsing a particular product. However when immunoaffinity columns are being used the performance of the method is dependant on, and thus linked to, the specific column being tested. In table VI

the $RSD_R$ values, for the total aflatoxin levels in the samples, are compared with those from previous trials using the Biocode immunoaffinity column. The $RSD_R$ values for the total aflatoxin levels, for the Aflaprep column, ranged from 30-54%. These were compared with the $RSD_R$ values from the Biocode column trials, which were 33-46%. All these trials analysed samples containing a similar range of aflatoxin contamination. From these results it can be concluded that there is no significant difference in $RSD_R$ values for each of the columns that have been subject to collaborative tests for the determination of aflatoxin levels in peanut butter.

The samples which were analysed showed an unusual pattern of aflatoxin contamination in so far as the major compound present was aflatoxin $G_1$. As far as we know no collaborative trial has been undertaken on samples which were so contaminated, all previous trials analysing material principally contaminated with aflatoxin $B_1$. The samples, after preparation and before the trial took place, were analysed by BFMIRA using aqueous acetonitrile extraction, the Biocode clean-up procedure and HPLC (method based on reference 5). Mean results for the four contaminated samples were, respectively, 7.0, 9.2, 33.8 and 45.0 µg/kg total aflatoxin. These results were similar to the results obtained for each respective sample in this collaborative trial and, as the majority of contamination in these samples was from aflatoxin $G_1$, it can be suggested, even though this work with the Biocode column was carried out by only one laboratory, that the antibody present in the Aflaprep column has an equivalent affinity for aflatoxin $G_1$ than does the antibody used in the Biocode columns.

**Conclusion:**

For the analysis of aflatoxin contaminated peanut butter the method described in this report (using the Aflaprep column) will give analytical results of similar precision as the method described in the previous collaborative trials (using the Biocode column). This trial was undertaken by laboratories which undertake a wide spectrum of food analysis work and not all of which analyse aflatoxins regularly. If a further collaborative trial was organised on an international level with expert laboratories to test the Aflaprep method it may be anticipated that lower $RSD_R$ values than those obtained here may be achieved.

## Appendix I

## Quantitative Determination by HPLC of Aflatoxins present in Peanut Butter using AFLAPREP Immunoaffinity Chromatography Sample Clean-up

### 1. Scope And Field Of Application

The method is designed to evaluate the Aflaprep immunoaffinity column for the determination of aflatoxins in peanut butter.

### 2. Definition

The content of aflatoxin: the concentrations of the aflatoxins $B_1$, $B_2$, $G_1$ and $G_2$ in peanut butter in µg/kg as determined by the method.

### 3. Principle

The aflatoxins are extracted from the samples with a methanol-water mixture. A portion of the diluted extract is passed through the immunoaffinity column. The column is washed to remove non-specific interfering substances followed by

elution of aflatoxins from the affinity column. Individual and, hence, total aflatoxins can be quantitated by HPLC determination of the eluate.

## 4. Reagents

**4.1** Sodium Chloride (Analytical Grade).

**4.2** Methanol. Technical grade is suitable for the extraction solvent which consists of methanol : water (70:30 v/v). HPLC grade methanol is required for elution, preparation of the aflatoxin standards and the HPLC mobile phase.

**4.3** Acetonitrile (Analytical Grade).

**4.4** HPLC Mobile Phase. This solution consists of distilled water : acetonitrile : methanol (50:30:20 v/v/v).

**4.5** HPLC Postcolumn Reagent (saturated iodine in water).

**4.6** Aflatoxin Standards. Crystalline powder of aflatoxins $B_1$, $B_2$, $G_1$ and $G_2$ is resuspended in benzene : acetonitrile (98:2 v/v) to give a stock concentrate solution containing 1mg/ml. To prepare the aflatoxin standard working solution first dilute 50µl of the respective toxin stock concentrate to 5ml with methanol. Measure the absorbance of the toxin solutions in a U.V. spectrophotometer, set at 362nm, and blank against methanol.

Dilute each toxin solution with methanol to give 100ng/ml. Mix 1ml aliquots of the toxin solutions to give a mixture of 100ng total aflatoxins per 1ml methanol. To prepare the HPLC standard solution, dilute 1ml 100ng/ml total aflatoxins with 4ml acetonitrile : water (15:25 v/v). Inject 200µl standard solution into the HPLC to produce 4 peaks: each peak height or area is equivalent to 1ng toxin. The elution order is $G_2$, $G_1$, $B_2$, and $B_1$.

## 5. Apparatus

**5.1** Laboratory Balance. Working range 1 - 100g, capable of accuracy to 1mg.

**5.2** Blender. A high quality waring blender or equivalent incorporating a high speed motor, complete with 1 litre heat resisting glass container with lid and stainless steel cutter.

**5.3** Filters. Whatman filter papers, 25cm, qualitative, grade 4. Whatman glass microfibre filters, 11cm.

**5.4** U.V. Spectrophotometer.

**5.5** Laboratory Glassware. Filter funnels; measuring cylinders for measuring 15ml, 30ml, and 125ml volumes; beakers (100-250ml capacity) for collecting sample filtrates and waste materials, 10ml amber vials for collecting eluates.

**5.6** Clampstand.

**5.7** Pipettes. Calibrated pipettes to cover the range 50-1000µl.

**5.8** HPLC System. A typical system to be used was: 2 x Waters M501 pumps or equivalent to deliver (a) mobile phase at a flow rate of 0.8ml/min and (b) saturated iodine solution at a flow rate of 0.4ml/min. Waters U6K universal injector or equivalent to introduce 50-200µl sample volumes onto the column. Hichrom guard column - Spherisorb ODS1, 5µm, 5cm x 5mm. Hichrom analytical column - Spherisorb ODS1, 5µm, 25cm x 5mm. Mixing T to introduce postcolumn reagent into column effluent and a reaction coil of 5ft. x 0.02 inch stainless steel tubing immersed in a water bath at 70°C. Waters 420 AC fluorescence detector or equivalent with 365nm excitation filter and 430nm emission filter. 5.9 Aflatest Kit Components. 25 immunoaffinity columns, 1 glass syringe barrel (10ml), 1 plastic syringe (pump unit), 1 rubber connector.

Table I
Aflatoxin level - ppb - Sample 1 (blank)

| Laboratory No | Aflatoxin B1 | | Aflatoxin G1 | | Total Aflatoxin (B1,B2,G1,G2) | |
|---|---|---|---|---|---|---|
| 1 | 0.00 | 0.51 | 0.00(a) | 0.86(a) | 0.00(a) | 1.37(a) |
| 2 | 0.00 | 0.00 | 0.00 | 0.00 | 0.00 | 0.00 |
| 3 | 0.45 | 0.52 | 7.00(b) | 7.30(b) | 7.45(b) | 7.82(b) |
| 4 | 0.50 | 0.50 | 0.50(a) | 0.70(a) | 1.40(b) | 1.40(b) |
| 5 | 0.00 | 0.00 | 0.00 | 0.00 | 0.00 | 0.00 |
| 6 | 0.40 | 0.20 | 0.00 | 0.00 | 0.40 | 0.20 |
| 7 | 0.00 | 0.00 | 0.00 | 0.00 | 0.00 | 0.00 |
| 8 | 0.60 | 0.60 | 0.00 | 0.00 | 0.60 | 0.60 |
| 9 | 0.00 | 0.00 | 0.00 | 0.00 | 0.00 | 0.00 |
| 10 | 0.10 | 0.40 | 0.00 | 0.00 | 0.10 | 0.40 |
| 11 | 0.00 | 0.60 | 0.00(a) | 9.60(a) | 0.00(a) | 10.5(a) |
| 12 | 0.00 | 0.00 | 0.00 | 0.00 | 0.00 | 0.00 |
| 13 | 0.40 | 0.00 | 0.00 | 0.00 | 0.40 | 0.00 |
| 14 | 0.20 | 0.30 | 0.20(b) | 0.20(b) | 0.40 | 0.50 |
| 15 | 0.30 | 0.20 | 0.00(a) | 0.30(a) | 0.50 | 0.60 |
| 16 | 0.00 | 0.00 | 0.00 | 0.00 | 0.00 | 0.00 |
| 17 | 1.8(a) | 0.00(a) | 0.00 | 0.00 | 1.80(a) | 0.00(a) |
| 18 | 0.00 | 0.00 | 0.00 | 0.00 | 0.00 | 0.00 |
| 19 | 0.40 | 0.40 | 0.00 | 0.00 | 0.40 | 0.40 |
| 20 | 0.00 | 0.00 | 0.00 | 0.00 | 0.00 | 0.00 |
| 21 | 0.00 | 0.00 | 0.00 | 0.00 | 0.00(a) | 0.70(a) |
| Mean | 0.19 | | 0.00 | | 0.19 | |
| r | | | | | | |
| $SD_r$ | | | | | | |
| $RSD_r$ % | | | | | | |
| R | | | | | | |
| $SD_R$ | | | | | | |
| $RSD_R$ % | | | | | | |

Table II
Aflatoxin level - Sample 2

| Aflatoxin B1 | | Aflatoxin G1 | | Total Aflatoxin (B1,B2,G1,G2) | |
|---|---|---|---|---|---|
| 1.90 | 0.66 | 10.30 | 5.93 | 12.20 | 6.59 |
| 0.90 | 0.80 | 4.40 | 4.10 | 5.80 | 4.90 |
| 1.27 | 1.05 | 12.00 | 10.10 | 14.27 | 12.55 |
| 1.20 | 1.20 | 7.00 | 6.40 | 8.90 | 8.50 |
| 3.70(a) | 0.00(a) | 8.14 | 0.00 | 11.84 | 0.00 |
| 0.90 | 1.20 | 5.60 | 6.60 | 7.00 | 8.20 |
| 0.00 | 0.00 | 0.00 | 4.29 | 0.00 | 4.29 |
| 1.70 | 1.60 | 8.00 | 8.00 | 10.10 | 10.00 |
| 0.00 | 1.20 | 5.10 | 2.50 | 5.10 | 3.70 |
| 0.60 | 0.90 | 5.10 | 4.40 | 7.62 | 7.06 |
| 1.30 | 0.90 | 6.10 | 4.90 | 7.70 | 5.80 |
| 0.70 | 1.70 | 6.50 | 6.10 | 7.60 | 8.50 |
| 1.40 | 1.40 | 7.20 | 7.60 | 10.00 | 10.30 |
| 1.00 | 0.20 | 5.30 | 0.80 | 6.70 | 1.00 |
| 0.80 | 1.10 | 4.70 | 4.50 | 6.00 | 6.10 |
| 0.00 | 0.00 | 2.90 | 7.60 | 2.90 | 10.10 |
| 2.10 | 0.60 | 10.30 | 1.40 | 12.40 | 2.00 |
| 0.00 | 0.00 | 0.00 | 0.00 | 0.00 | 0.00 |
| 0.90 | 1.10 | 4.20 | 4.60 | 7.10 | 7.50 |
| 1.60 | 1.50 | 8.30 | 6.50 | 9.90 | 9.40 |
| 7.20(a) | 12.50(a) | 19.60(b) | 22.20(b) | 26.80(b) | 38.70(b) |
| 0.93 | | 5.44 | | 6.99 | |
| 1.24 | | 6.90 | | 8.80 | |
| 0.44 | | 2.46 | | 3.14 | |
| 47.45 | | 45.32 | | 44.96 | |
| 1.65 | | 8.32 | | 10.47 | |
| 0.59 | | 2.97 | | 3.74 | |
| 63.43 | | 54.68 | | 53.51 | |

**For Key, see Table VII**

## 6. Procedure

**6.1** Sample preparation and extraction. Weigh 25g sample, 5g NaCl, and add 125ml methanol : water (70/30, v/v) into blender jar, cover and blend for 1 min at high speed. Immediately after mixing, pass 25-30ml sample extract through Whatman No. 4 filter paper. Measure 15ml filtrate and 30ml distilled water into a small beaker and mix. Refilter through glass microfibre filter immediately before passing through immunoaffinity column.

**6.2** Unit Assembly. Aflatest immunoaffinity columns should be at ambient temperature prior to use. Ensure that the column has not dried out and contains buffer above the resin. Remove the cap from the top of the column, cut off the sealed end and replace on the column. Firmly attach the column to the 10ml glass syringe barrel and place in the clampstand. Ensure that the bottom plug of the affinity column is still in place.

Table III

Aflatoxin Level - Sample 3

| Laboratory No | Aflatoxin B1 | | Aflatoxin G1 | | Total Aflatoxin (B1,B2,G1,G2) | |
|---|---|---|---|---|---|---|
| 1 | 2.12 | 2.63 | 10.69 | 13.65 | 12.81 | 16.98 |
| 2 | 1.10 | 1.10 | 6.70 | 6.00 | 8.40 | 7.50 |
| 3 | 1.42 | 1.65 | 12.00 | 12.80 | 14.52 | 15.55 |
| 4 | 1.80 | 1.80 | 10.80 | 11.00 | 13.50 | 13.70 |
| 5 | 3.53(a) | 0.00(a) | 8.58 | 10.51 | 12.11 | 10.51 |
| 6 | 1.60 | 1.50 | 10.40 | 9.90 | 13.00 | 12.10 |
| 7 | 0.00 | 0.00 | 18.79(a) | 6.24(a) | 18.79(a) | 12.51(a) |
| 8 | 2.20 | 3.00 | 11.70 | 15.50 | 14.40 | 19.20 |
| 9 | 22.40(a) | 0.00(a) | 30.10(a) | 7.80(a) | 53.80(a) | 7.80(a) |
| 10 | 1.50 | 1.00 | 9.70 | 6.00 | 14.16 | 9.40 |
| 11 | 1.60 | 1.90 | 9.60 | 10.70 | 11.50 | 12.90 |
| 12 | 1.50 | 1.30 | 8.00 | 5.70 | 11.20 | 7.90 |
| 13 | 1.80 | 2.00 | 11.80 | 12.00 | 15.20 | 15.60 |
| 14 | 0.00 | 1.50 | 0.00 | 8.70 | 5.80 | 10.60 |
| 15 | 1.30 | 1.20 | 7.20 | 7.80 | 9.30 | 9.80 |
| 16 | 0.00 | 1.60 | 3.40 | 6.70 | 3.40 | 8.30 |
| 17 | 1.40 | 1.40 | 10.20 | 8.90 | 11.60 | 10.30 |
| 18 | 1.80 | 0.00 | 5.30 | 4.50 | 7.10 | 4.50 |
| 19 | 1.40 | 1.30 | 6.80 | 6.70 | 10.80 | 10.40 |
| 20 | 1.30 | 1.40 | 8.70 | 9.80 | 11.50 | 11.20 |
| 21 | 13.50(b) | 9.20(b) | 30.00(b) | 29.60(b) | 43.50(b) | 38.80(b) |
| Mean | 1.39 | | 9.11 | | 11.30 | |
| r | 1.44 | | 3.82 | | 5.47 | |
| $SD_r$ | 0.51 | | 1.37 | | 1.95 | |
| $RSD_r$ % | 36.83 | | 14.99 | | 17.29 | |
| R | 1.96 | | 7.84 | | 9.65 | |
| $SD_R$ | 0.70 | | 2.80 | | 3.45 | |
| $RSD_R$ % | 50.28 | | 30.74 | | 30.49 | |

Table IV

Aflatoxin Level - Sample 4

| Laboratory No | Aflatoxin B1 | | Aflatoxin G1 | | Total Aflatoxin (B1,B2,G1,G2) | |
|---|---|---|---|---|---|---|
| 1 | 8.54 | 7.50 | 52.34 | 30.26 | 62.27 | 38.86 |
| 2 | 4.20 | 4.10 | 22.20 | 21.50 | 29.10 | 27.80 |
| 3 | 2.32 | 2.02 | 17.20 | 15.10 | 23.22 | 20.72 |
| 4 | 5.80 | 6.00 | 36.00 | 36.50 | 43.70 | 45.30 |
| 5 | 4.13 | 3.53 | 22.42 | 20.72 | 26.55 | 24.25 |
| 6 | 4.80 | 4.70 | 29.70 | 29.30 | 37.60 | 37.00 |
| 7 | 0.00 | 2.83 | 7.25(a) | 44.53(a) | 7.63(a) | 48.81(a) |
| 8 | 8.90 | 9.60 | 45.50 | 47.50 | 56.20 | 59.10 |
| 9 | 2.50 | 1.60 | 12.20 | 14.60 | 15.90 | 16.20 |
| 10 | 2.40 | 6.70 | 11.90 | 19.00 | 18.38 | 27.62 |
| 11 | 5.40 | 5.20 | 31.40 | 31.20 | 38.00 | 37.60 |
| 12 | 4.90 | 4.10 | 21.00 | 17.50 | 28.20 | 24.40 |
| 13 | 5.70 | 5.40 | 34.90 | 35.70 | 46.20 | 46.60 |
| 14 | 4.50 | 4.30 | 26.50 | 24.80 | 31.90 | 29.90 |
| 15 | 7.40 | 4.00 | 37.00 | 21.70 | 47.30 | 28.30 |
| 16 | 4.00 | 1.70 | 33.00 | 14.00 | 39.00 | 18.30 |
| 17 | 4.00 | 3.60 | 24.50 | 19.00 | 28.50 | 22.60 |
| 18 | 3.60 | 3.40 | 19.90 | 19.60 | 25.20 | 23.00 |
| 19 | 3.80 | 4.70 | 20.20 | 22.10 | 28.10 | 32.10 |
| 20 | 5.20 | 4.00 | 33.40 | 27.20 | 41.60 | 33.90 |
| 21 | 19.80(b) | 22.50(b) | 67.80(b) | 99.60(b) | 87.60(b) | 122.10(b) |
| Mean | 4.53 | | 26.28 | | 33.17 | |
| r | 3.11 | | 15.99 | | 18.06 | |
| $SD_r$ | 1.11 | | 5.71 | | 6.45 | |
| $RSD_r$ % | 24.52 | | 21.73 | | 19.45 | |
| R | 5.67 | | 27.56 | | 32.92 | |
| $SD_R$ | 2.03 | | 9.84 | | 11.76 | |
| $RSD_R$ % | 44.73 | | 37.46 | | 35.44 | |

**For Key, see Table VII**

**6.3** Immunoaffinity Chromatography. Remove the bottom plug from the immunoaffinity column and pass 15ml sample filtrate (equivalent to 1g original sample) slowly through the column using the hand pump. A flow rate of 2-3ml/min is strongly recommended. Collect the waste in a suitable container. Wash the column 2 times with 10ml distilled water. Ensure that all the residual water has been expelled from the column before the next stage. Place a 10ml amber collection vial directly beneath the column. Pipette 1ml methanol : acetonitrile (20:30 v/v) into the glass syringe barrel. Elute aflatoxins from the column by slowly passing the eluant through the column at a flow rate of 1 drop per second. Back flushing with the eluant 2-3 times is recommended to ensure complete denaturation of the monoclonal antibody with the subsequent release of aflatoxins into solution. Pipette 1ml distilled water into the glass syringe barrel, pass through the column and collect in the amber vial, to give a 2ml total volume.

## Table V

Aflatoxin Level - Sample 5

| Laboratory No | Aflatoxin B1 | | Aflatoxin G1 | | Total Aflatoxin (B1,B2,G1,G2) | |
|---|---|---|---|---|---|---|
| 1 | 8.61 | 10.58 | 33.10 | 47.11 | 41.71 | 59.33 |
| 2 | 5.50 | 5.50 | 28.10 | 28.20 | 36.50 | 36.70 |
| 3 | 3.67 | 1.50 | 25.70 | 11.10 | 34.87 | 17.00 |
| 4 | 7.40 | 7.60 | 47.70 | 49.40 | 57.90 | 59.60 |
| 5 | 7.48 | 5.85 | 35.52 | 33.82 | 43.00 | 39.67 |
| 6 | 7.20 | 6.80 | 48.20 | 43.30 | 59.60 | 53.90 |
| 7 | 4.10 | 3.05 | 90.07 | 58.87 | 95.04 | 63.54 |
| 8 | 12.40 | 11.50 | 68.00 | 61.90 | 82.90 | 75.90 |
| 9 | 0.00 | 3.50 | 15.80 | 18.50 | 16.70 | 24.10 |
| 10 | 7.60 | 7.90 | 26.40 | 33.90 | 39.12 | 55.96 |
| 11 | 7.70 | 7.40 | 47.20 | 44.30 | 56.30 | 53.10 |
| 12 | 7.80 | 5.00 | 26.00 | 26.00 | 36.40 | 34.70 |
| 13 | 6.70 | 8.30 | 48.80 | 59.40 | 62.60 | 78.40 |
| 14 | 7.10 | 5.80 | 37.30 | 34.40 | 46.70 | 42.30 |
| 15 | 7.00 | 6.00 | 38.60 | 30.20 | 48.50 | 39.30 |
| 16 | 3.40 | 5.60 | 17.00 | 45.00 | 22.70 | 55.40 |
| 17 | 5.20 | 5.80 | 34.70 | 44.60 | 39.90 | 50.40 |
| 18 | 4.30 | 3.80 | 21.90 | 24.40 | 27.90 | 29.80 |
| 19 | 6.30 | 6.90 | 29.70 | 33.80 | 43.60 | 46.50 |
| 20 | 5.70 | 5.60 | 41.60 | 40.30 | 51.40 | 50.00 |
| 21 | 36.50(a) | 28.70(a) | 161.40(b) | 155.90(b) | 200.10(b) | 185.80(b) |
| Mean | | 6.23 | | 38.25 | | 47.72 |
| r | | 2.96 | | 22.66 | | 26.62 |
| $SD_r$ | | 1.06 | | 8.09 | | 9.51 |
| $RSD_r$ % | | 16.97 | | 21.16 | | 19.92 |
| R | | 6.82 | | 43.77 | | 48.29 |
| $SD_R$ | | 2.43 | | 15.63 | | 17.24 |
| $RSD_R$ % | | 39.08 | | 40.87 | | 36.14 |

**For Key, see Table VII**

## Table VI

| **Collaborative Trial** | **Biocode trial U.K.** | | **Biocode International Trial** | | | **Aflaprep - May & Baker U.K.Trial** | | | |
|---|---|---|---|---|---|---|---|---|---|
| Aflatoxin level in sample (µg/kg) | 12 | 35 | 4 | 15 | 38 | 7 | 11 | 33 | 48 |
| $RSD_R$ % | 46 | 36 | 33 | 45 | 42 | 54 | 30 | 35 | 36 |

**6.4** Quantitation of Aflatoxins by HPLC. Following start-up and equilibration of HPLC system, inject 200µl aflatoxin standard solution onto HPLC. The elution order is $G_2$, $G_1$, $B_2$ and $B_1$; each peak height or area is equivalent to 1ng toxin. Inject 200µl test sample (equivalent to 0.1g original sample) onto HPLC. Quantitate aflatoxin concentrations by comparing sample peak heights or areas to the standards.

## 7. Precautions

Aflatoxins are very hazardous substances. Rubber gloves, safety glasses and laboratory coats should be worn throughout the assay. All materials and reagents must be decontaminated by soaking for at least 2 hours in a solution of sodium hypochlorite (10% v/v), prior to rinsing thoroughly with distilled water. It is recommended that the glass syringe barrel is rinsed through with methanol and then distilled water at the end of each assay.

## 8. Expression Of Results

**8.1** Calculation of aflatoxins: The concentration of aflatoxins, in µg/kg is given by:

$$\text{(AFLATOXIN), (µg/kg)} = \frac{\text{peak ht. or area of sample} \times 10}{\text{peak ht. or area of standard}}$$

Add the concentrations of the four aflatoxins to obtain total aflatoxin concentration.

**8.2** Repeatability: To be assessed from the results of the collaborative trial.

**8.3** Reproducibility: To be assessed from the results of the collaborative trial.

**8.4** Relative standard deviation for reproducibility: To be assessed from the results of the collaborative trial.

TABLE VII

| | |
|---|---|
| r | Repeatability (within-lab variation). The value below which the absolute difference between two single test results obtained under the same method on identical test material under the same conditions may be expected to lie with 95 % probability. |
| R | Reproducibility (between-lab variation). The value below which the absolute difference between two single test results obtained under the same method on identical test material under different conditions may be expected to lie with 95 % probability. |
| $SD_r$ | The standard deviation of the repeatability. |
| $SD_R$ | The standard deviation of the reproducibility |
| $RSD_r$ | The relative standard deviation of the repeatability $SD_r \times 100 / \bar{x}$ ($\bar{x}$ = mean) |
| $RSD_R$ | The relative standard deviation of the reproducibility $SD_R \times 100 / \bar{x}$ ($\bar{x}$ =mean) |
| (a) | Result rejected as outlier by Cochran's test ($p < 0.05$) values not used in calculation of mean, repeatability, reproducibility and relative standard deviation of repeatability and reproducibility |
| (b) | Result rejected as outlier by Grubb's test ($p < 0.05$) values not used in calculation of mean, repeatability, reproducibility and relative standard deviation of repeatability and reproducibility |

## Acknowledgements

The authors thank the following collaborators and their staff, for participating in the trial:

R.E.S. Andrews, Kent County Council, Maidstone;
R.J.C. Barron, Clayton Bostock Hill & Rigby, Birmingham;
H.M. Bee, County Analyst's Department, Stafford;
M.J. Billington, Birmingham Technical & Scientific Complex, Birmingham;
K.T. Chisnall, Derbyshire County Laboratory, Matlock;
E.C. Conchie, Ruddock & Sherratt, Manchester;
D. Dunn, A.H. Allen & Partners, Sheffield;
J. Duszanskyj, KP Foods, Leicestershire;
M.F. Godfrey, Lothian Regional Council, Edinburgh;
P. Hall, Dr. Bernard Dyer and Partners Ltd, London;
G. Keen, Hereford & Worcester County Laboratory, Worcester;
E.E.J. King, The Hampshire Scientific Service, Southsea;
D.W. Lord, Lancashire County Laboratory, Preston;
E.B. Reynolds, Tickle & Reynolds, Exeter;
D.G. Rixon, Mid Glamorgan County Public Health Laboratory, Cardiff;
B. Sanders, Cardiff City Analysts Laboratory;
T. D'Silva, Laboratory of the Government Chemist, London;
R.A. Stevens, Southwark Analytical and Scientific Services, London;
B. Taylor, Greater Manchester Scientific Services, Didsbury;
J.S. Turnball, Avon County Scientific Services, Bristol;
J.P. Wootten, Central Scientific Laboratories, London.
Also thanked are G.M. Wood and P.J. Mann (BFMIRA) for analytical investigation and C. Clark (May & Baker Diagnostics Ltd, Glasgow) for donation of columns and protocol.

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**2.** Busby, W.F.Jr and Wogan G.N, 1984, Aflatoxins. Chemical Carcinogens, edited by C.E.Searle (Washington D C : American Chemical Society), 945 - 1136.

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**7.** "Protocol for the Design, Conduct and Interpretation of Collaborative Studies" Ed W Horwitz, Pure and Appl Chem., 1988, **60(6)**, 855 - 864.

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J. Assoc. Publ. Analysts, 1992, 28, 11-16

# MAFF VALIDATED METHODS FOR THE ANALYSIS OF FOODSTUFFS [1]

## No. V0
## INTRODUCTION, GENERAL CONSIDERATIONS AND ANALYTICAL QUALITY CONTROL

## Introduction

The European Community (EC) is laying down rules for food analysis which will aid the operation of the Single Market. Firm guidelines have been adopted which define the criteria to be considered when a method of analysis is to be included in a foodstuffs Directive[(1)] These criteria include specificity, accuracy, precision, sensitivity, practicability and applicability; they are similar to those required by the Codex Alimentarius Commission[(2)]. In particular, precision parameters such as repeatability (r) and reproducibility (R) are required, and these must be determined by collaborative trial. General agreement on many aspects of the conduct of such trials has now been reached as a result of the development of the collaborative trial protocol of the International Union of Pure and Applied Chemistry (IUPAC)[(3)]; the way now lies open towards recognition of analytical protocols and hence one element in the acceptance of analytical results across the EC.

Other elements include demonstration of laboratory quality standards; the availability of accepted methods of analysis will not diminish the need for individual laboratories to demonstrate their competence on a continuing basis via accreditation and participation in proficiency testing schemes.

Before a food analysis procedure can be acceptable for legislative purposes, an appropriate method must be selected, its protocol standardised and its performance characteristics established. In order to promote harmonisation, the Ministry of Agriculture, Fisheries and Food (MAFF) has developed a collaborative trial programme to validate methods of analysis for food. The reports of these collaborative trials are normally published in the scientific literature; in addition methods successfully validated will be published within the Journal of the Association of Public Analysts.

This Series may also include methods of particular interest which have been validated by a collaborative trial not carried out under the direct auspices of the MAFF collaborative trial programme.

The performance characteristics of the methods, and guidance as to their interpretation, are presented in the validated methods series separately

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0004-5780/93 +6 $020.00

from the protocol text and in the form recommended by IUPAC[4].General procedural considerations and principles of Analytical Quality Control are given in Protocol V0 of the series and also the introductory booklet, and apply to all methods: specific applications are covered individually with each method.

Although methods validated in this Series will not have any specific legal status in food law enforcement, their reliability and authority will make them invaluable to both the food industry and to enforcement authorities.

In addition, a further series of booklets is being prepared, "Statutory Methods" (S1, S2, etc.), which will give the principle and references for those methods of analysis and examination which are already part of UK legislation for foodstuffs.

## General Considerations

### 1 Preparation of the Analysis Sample

**1.1** The mass of the sample presented to the laboratory for analysis shall be sufficient to enable all the determinations required of the sample to be carried out.

**1.2** Mixing

The sample for analysis shall always be mixed thoroughly before any test portion is weighed out. Samples in powder or paste form shall be removed from the container, any lumps broken down, the sample mixed in an appropriate manner and placed in a suitable container. Samples in liquid form shall be mixed by stirring.

**1.3** Containers

The sample shall always be kept in an airtight and moisture-tight container, at a temperature which ensures that no change takes place which would affect the result of the analysis.

### 2 Reagents

**2.1** Water

Wherever mention is made of water for solution, dilution or washing purposes, distilled water, or demineralised water of at least equivalent purity, shall be used.

**2.2** Solvent

Wherever reference is made to "solution" or "dilution" without further indication, "solution in water" or "dilution with water" is meant.

**2.3** Chemicals

All chemicals shall be of recognised analytical reagent grade quality except where otherwise specified.

## 3 Equipment

The lists of apparatus contain only those items with a specialised use and items with a particular specification. "Analytical balance" means a balance capable of weighing to a precision of at least 0.1 mg.

## 4 Expression of Results

The result stated in the analytical report shall be the mean value obtained from at least two determinations, the repeatability of which is satisfactory. It shall not contain more significant figures than are justified by the precision of the method of analysis used. Except where otherwise specified, the result shall be calculated as a percentage by mass of the sample.

## 5 Test Report

The test report shall identify the method of analysis used (with references where appropriate) as well as the results obtained. In addition, it shall mention all details of procedure, not specified in the method of analysis, or which are optional, as well as any circumstances that may have influenced the results obtained.

The test report shall give all the information necessary for the complete identification of the sample.

# ANALYTICAL QUALITY CONTROL

## GENERAL PRINCIPLES

## 1 Repeatability

When a check on the precision of analysis within a laboratory is required, two test results for each sample must be obtained under conditions of repeatability, i.e. conditions where independent observed values or test results are obtained with the same method on identical test material, in the same laboratory by the same operator using the same equipment, within the shortest feasible interval of time. Each protocol gives observed values of repeatability, r, for the method it describes, and suggests a practicable target for the repeatability, r.

The term 'single test result' shall be the value obtained when the standardised test method is applied fully and once to a single sample.

## 2 Acceptability of Test Results

When two test results are obtained under conditions of repeatability, the final quoted result is their mean (e.g. arithmetic for chemical analysis, geometric for food examination procedures), provided that the conditions for acceptable repeatability are met. If this is not the case, the cause

should be investigated; if there is no obvious cause, further results must be obtained until the overall repeatability becomes acceptable.

**2.1** Procedure for assessing acceptability

**2.1.1** If the absolute difference between two test results $x_1$ and $x_2$ is less than r, the precision is acceptable, and the quoted result is their mean. If not, the procedure is continued (**2.1.2**).

**2.1.2** A third result $x_3$ is obtained. If the absolute difference between the highest and lowest values of x is less than 1.2r, the precision is acceptable, and the quoted result is the mean of all three values of x. If not, the procedure is continued (**2.1.3**).

**2.1.3** A fourth result $x_4$ is obtained. If the absolute difference between the highest and lowest values of x is less than 1.3r, the precision is acceptable, and the quoted result is the mean of all four values of x. If not, the precision is questionable but the quoted result should be the median of the four values of x, i.e. the mean of the middle two values.

## 3 Reproducibility

When a check on the precision of analysis in two or more laboratories is required, two test results for each sample must be obtained under conditions of reproducibility, i.e. conditions where observed values or test results are obtained with the same method on identical test material in different laboratories with different operators using different equipment. Each protocol gives observed values of R for the method it describes, and suggests a practicable target for the reproducibility, R. It should be noted that the values of reproducibility (R) apply in the particular case where a single test result from each laboratory is compared. If it is desired to compare final quoted test results, which are the mean results from two or more "single test results" carried out by each laboratory on identical laboratory samples, the appropriate values for R may be calculated as outlined in ISO 5725 Part 6 Section 2.

## 4 Trueness ( Bias )

When a check on the trueness of the test results is required, one or more reference materials (certified or prepared in- house), of similar matrix and analyte concentration to that of the test samples, must be analysed in parallel with the test samples. A decision is then made as to whether the difference, if any, between the expected value(s) for the reference material(s) and that obtained by analysis of the latter is statistically significant. With certified reference materials, confidence limits are cited; prepared in- house reference materials need plotted data such as those on control charts. In the latter case, a test result obtained for the prepared reference material which falls within ±2 units of the standard deviation (S) of the accepted mean value for the analyte of interest may be considered acceptable. Here, the accepted mean value and standard deviation would be calculated from the results obtained from not less than

ten analyses of the prepared in-house reference material. If the difference between the test result and the mean value exceeds ±3S, the test result must be rejected.

In appropriate cases, a check on consistent method and individual laboratory bias may be made by spiking and determining the recovery of the added analyte.

## 5 Limit of Detection

Recommendations have been made by various organisations on the determination of detection limits (e.g. by the Analytical Methods Committee of the Royal Society of Chemistry)[5]. In this series of papers the lower limit of precise determination, or of absolute detection, can only be estimated by analysing samples with known low levels of analyte. In the absence of such data, the limiting concentration has been taken to be the value of the repeatability, r, extrapolated to such levels.

## 6 Interpretation of Observed Levels

Each protocol tabulates the statistical parameters observed in the collaborative trial of the method: these include the mean observed level of analyte, the repeatability (r), the reproducibility (R), the corresponding standard deviations $S_r$ and $S_R$, and the corresponding relative standard deviations (coefficients of variation), RSD. The following relationships are employed: $R = 2.8S_R$; $r = 2.8S_r$; RSD = 100S/mean.

## 7 COSHH

**No information has been given on COSHH (Control of Substances Hazardous to Health) aspects of the methods. Analysts must make the appropriate hazard and risk assessments required by COSHH before using the methods in their own laboratories.**

## 8 Key to the Symbols and Definitions of the Statistical Parameters used in the Protocols

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

## References

1. Council Directive 85/591/EEC of 20th December, 1985, O. J. L 372, 31.12.85
2. Codex Alimentarius Commission Procedural Manual, Seventh Edition
3. "Protocol for the Design, Conduct and Interpretation of Collaborative Studies", Pure and Applied Chemistry, 1988, **60(6)**, 855-664
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J. Assoc. Publ. Analysts, 1992, 28, 17-24

# MAFF VALIDATED METHODS FOR THE ANALYSIS OF FOODSTUFFS

## No. V 1

## DIETARY FIBRE (COLORIMETRY)

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

### 1 Scope and field of application

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

### 2 Definition

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

### 3 Principle

The starch is gelatinised and then removed by enzymic digestion. The remaining polysaccharides are hydrolysed by sulphuric acid and the resulting sugars determined colorimetrically. Two alternative procedures are described for the dispersion of the starch thus enabling evaluation of the samples for dietary fibre as NSP alone and as NSP plus RS. The procedures are summarised in Appendix 1.

### 4 Reagents

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

**4.1** Acetone

**4.2** Dimethyl sulphoxide

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

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

**4.4.1** Sodium acetate, 0.1 mol/l: prepare by dissolving 13.6 g sodium acetate trihydrate, $CH_3COONa.3H_2O$, and making up to 1 litre with benzoic acid solution (**4.3**).

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

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

**4.4.4** Sodium acetate buffer, 0.1 mol/l: adjust sodium acetate 0.1 mol/l (**4.4.1**) to pH 5.2 with acetic acid, 0.1 mol/l (**4.4.2**). To stabilise and activate enzymes, add 4 ml of calcium chloride, 1 mol/l (**4.4.3**) to 1 litre of buffer.

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**4.5** α-Amylase, EC 32.1.1: Pancrex V Capsules (approximately 9000 BP units α-amylase per capsule, Paines and Bryne Ltd).

**4.5.1** α-Amylase solution, 2000 BP units/ml: empty 2 Pancrex V capsules (**4.5**) into a centrifuge tube. Add 9 ml of water and disperse using a vortex mixer. Centrifuge at 1500 g for 10 min. and use the supernatant as α-amylase solution. Prepare immediately before use.

**4.6** Pullulanase, 100 units/ml: EC 3.2.1.41 (Boehringer 108944)

**4.6.1** Pullulanase solution, 1 unit/ml: dilute pullulanase (**4.6**) 1:100 (eg 0.010 ml made up to final volume of 1 ml) with acetate buffer (**4.4.4**). Prepare immediately before use.

**4.7** Ethanol, absolute

**4.8** Ethanol, 85% (*V/V*).

**4.9** Sulphuric acid, 12 mol/l.

**4.10** Sulphuric acid, 2 mol/l.

**4.11** Glucose solution, 0.5 mg/ml: in 50% saturated benzoic acid solution (**4.3**).

**4.12** Sodium hydroxide

**4.13** Sodium hydroxide solution, 3.9 mol/l.

**4.14** Sodium potassium tartrate

**4.15** Dinitrosalicylate solution: dissolve 10 g of 3,5-dinitrosalicylic acid, 16 g of sodium hydroxide (**4.12**) and 300 g of sodium potassium tartrate (**4.14**) in re-distilled water and make up to 1 litre with this water. Store in a well-capped dark bottle. Keep for 2 days before use. The solution is stable for at least six months at room temperature.

**4.16** Standard sugar solution: dissolve 600 mg of arabinose, 800 mg of xylose and 600 mg glucose in benzoic acid solution (**4.3**) and make up to 500 ml with the benzoic acid solution to provide a stock solution. To prepare standards, take 1, 2, 3 and 4 ml of stock solution and make up to 4 ml with the benzoic acid. Add 4 ml of sulphuric acid, 2 mol/l (**4.10**), to give standards of 0.5, 1.0, 1.5 and 2.0 mg total sugars/ml in sulphuric acid 1 mol/l.

## 5 Apparatus:

**5.1** Centrifuge

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

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

**5.4** Magnetic stirrers: PTFE coated, to fit centrifuge tubes (**5.2**).

**5.5** Vortex mixer

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

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

**5.8** Oven, capable of maintaining a temperature of 42 ± 2°C for 24 hr.

## 6 Procedure

**6.1** Pre-treatment of samples

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

**6.2** Test samples

Accurately weigh, to the nearest 0.1 mg, two portions (a) and (b) of the sample, each between 100 and 500 mg (but containing no more than 200 mg of dry matter and 50 mg NSP, e.g. 200 mg flour, 100 mg bran) into a 50 ml screw-top centrifuge tube (**5.2**) and add a stirrer (**5.4**).

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

**6.3** Fat extraction

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

**6.4** Dispersion and enzymic hydrolysis

**6.4.1** Dispersion of the starch

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

Remove tube (a) from the beaker and immediately, without cooling, add 8 ml of acetate buffer (**4.4.4**), pre-equilibrated at 50 ± 5°C, and vortex mix. Then remove tube (b) from the beaker.

**6.4.2** Enzymic hydrolysis of starch

Note: All portions of the sample in their associated tubes are to be treated in the same manner in the procedure given in Section 6.4.2 through to Section 6.6 of the method. Reference to "a sample" or "a

tube" is taken to include "all samples" or all tubes" being analysed in this part of the method.

Leave the tubes at room temperature or in a water bath at 37°C, but only until the contents have cooled to between 30°C and 40°C and then immediately add 0.5 ml α-amylase solution (**4.5.1**) followed by 0.1 ml of pullulanase solution (**4.6.1**) and vortex-mix. (NB: do not combine the enzyme solutions before addition).

Incubate the sample at 42 ± 2°C (**5.6** or **5.8**) for 16 to 18 hr. Vortex mix after the first hour.

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

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

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

**6.5** Acid hydrolysis of the residue from enzymic digestion

Disperse the dried residue from Section 6.4.2 in 2 ml of sulphuric acid, 12 mol/l (**4.9**) with vortex-mixing. Leave at 35 ± 1°C (**5.6**) for 1 to 1.25 hr., with occasional mixing to disperse the cellulose. Rapidly add 22 ml of water and mix. Place in boiling water (**5.3**) for 2 to 2.25 hr. from re-boiling, stirring continuously. Cool to room temperature.

**6.6** Measurement of total reducing sugars

Place into separate test tube 1 ml of blank solution (Benzoic acid solution (**4.3**) and sulphuric acid solution, 2 mol/l, (**4.10**) diluted 0.5 ml + 0.5 ml), 1 ml of each of the standard solutions (**4.16**) and 1 ml of the hydrolysate (**6.5**). Add 0.5 ml glucose solution, 0.5 mg/ml (**4.11**) and 0.5 ml of sodium hydroxide, 3.9 mol/l (**4.13**) to each tube and vortex-mix.

Add 2 ml of dinitrosalicylate solution (**4.14**) to each tube and vortex-mix again.

Place the tubes, all at the same time, in a briskly boiling water-bath (**5.6**) for 10 min. Cool in water to room temperature. Add 20 ml of water and mix well by repeated inversion. Read the absorbance at 530 nm.

## 7 COSHH

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

## 8 Expression of Results

**8.1** Calculation of non-starch polysaccharides

The NSP content, in g/100g, is given by:

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

where:

$A_t$ is the absorbance of the test solution obtained from tube (a);
$V_t$ is the total volume (ml) of the test solution (here 24 ml);
$A_s$ is the absorbance corresponding to 1 mg sugar/ml taken from the line of best fit for the standard; and
$W_t$ is the weight (mg) of sample taken for analysis in tube (a).

**8.2** Calculation of non-starch polysaccharides with resistant starch

The NSP content together with resistant starch, in g/100g is given by:

$$\text{NSP+RS (g/100g)} = \frac{A_t}{A_s} \times \frac{V_t}{W_t} \times 100$$

where:

$A_t$ is the absorbance of the test solution obtained from tube (b);
$V_t$ is the total volume (ml) of the test solution (here 24 ml);
$A_s$ is the absorbance corresponding to 1 mg sugar/ml taken from the line of best fit for the standard; and
$W_t$ is the weight (mg) of sample taken for analysis in tube (b).

## 9 References

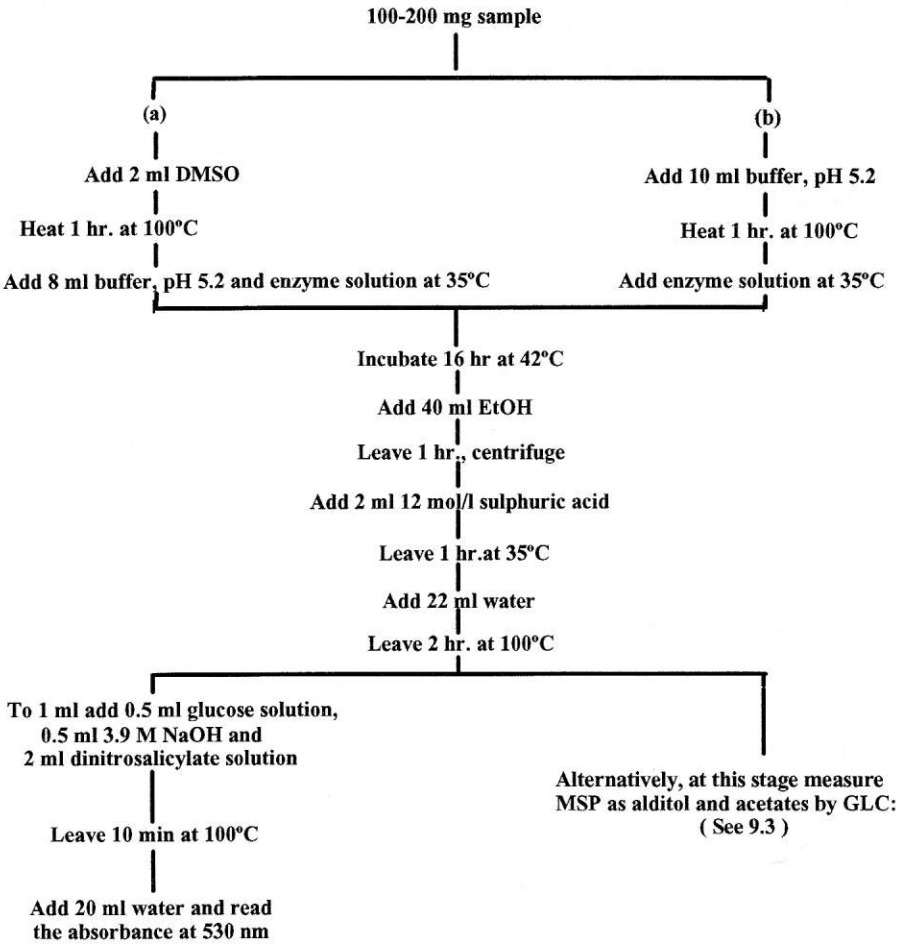
**9.1** H N Englyst, J H Cummings and R Wood J.Assoc. Publ. Analysts, 1987, **25**, 73-110

**9.2** H N Englyst and G J Hudson. Food Chem., 1987,**24**,63-76.

**9.3** MAFF Validated Methods for the Analysis of Foodstuffs. No V2; Dietary Fibre, Englyst Method: GLC End-point Determination. London, 1990.

## APPENDIX 1

## SCHEMATIC DIAGRAM FOR ANALYSIS OF DIETARY FIBRE




## APPENDIX 2

### Analytical Quality Control

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

### A1 Repeatability

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

## A2 Reproducibility

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

## A3 Trueness (Bias)

The trueness of the results cannot be estimated directly in the absence of pure analyte and of reference materials. Comparison of mean values from wholemeal and white breads and a mixture of the two indicates consistent recoveries during the collaborative trial. All the mean values of NSP and NSP plus RS observed during the trial agree quantitatively with those obtained by the full Englyst procedure (**9.3**), which may be regarded as a reference procedure only because it gives more detailed information of the specific sugar composition of the dietary fibre, after analysis by GLC. In this sense the full and simplified procedures are both accurate in the determination of dietary fibre as defined in Section 2 of this protocol, but observed values do not necessarily agree with results using other methods and other types of sample. in particular, they may not agree with the true levels of polysaccharide that escapes assimilation by an animal during digestion, though they are designed to reflect this. It is recommended that analytical data should normally be based on NSP alone, since RS is usually a minor constituent, it depends on the processing history of the sample and its physiological significance is uncertain.

The determination of RS alone, by subtracting NSP from NSP + RS (calculated as in Section 8.1 of the protocol) introduces excessive variability and cannot be recommended. The method is not precise enough to be suitable for the estimation by difference of the small levels of RS usually encountered.

## A4 Limit of Detection

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

## A5 Statistical Data: Derived from Results of Interlaboratory Tests

Participants in the collaborative trial at 19 laboratories each analysed a sample of wholemeal bread in duplicate as a pre-trial check. They then each analysed 14 samples once in the trial proper. These comprised 7 different samples (including the same wholemeal bread) in blind duplicate. The samples had been commercially prepared, ground and homogenised; they were artificially coloured to appear identical.

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

TABLE 1

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

| Sample | $\bar{x}$ | $S_r$ | $RSD_r$ | r | $S_R$ | $RSD_R$ | R |
|---|---|---|---|---|---|---|---|
| Wholemeal bread (pre-trial sample) | 10.59 | 0.46 | 4.4 | 1.29 | 1.07 | 10.1 | 3.00 |
| Wholemeal bread | 10.66 | 0.64 | 6.0 | 1.79 | 1.87 | 17.5 | 5.23 |
| Wholemeal/white bread (1:1) | 7.43 | 0.98 | 13.1 | 2.73 | 1.21 | 16.3 | 3.39 |
| White bread | 3.08 | 0.55 | 18.0 | 1.55 | 0.64 | 20.8 | 1.79 |
| High bran bread | 11.53 | 0.55 | 4.8 | 1.54 | 1.57 | 13.6 | 4.4 |
| Rye bread | 4.71 | 0.63 | 13.3 | 1.75 | 0.72 | 15.3 | 2.02 |
| Cornflakes | 1.09 | 0.33 | 29.8 | 0.91 | 0.41 | 37.4 | 1.14 |
| Oat cereal | 8.73 | 0.76 | 8.7 | 2.13 | 1.58 | 18.1 | 4.43 |

TABLE 2

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

| Sample | $\bar{x}$ | $S_r$ | $RSD_r$ | r | $S_R$ | $RSD_R$ | R |
|---|---|---|---|---|---|---|---|
| Wholemeal bread (pre-trial sample) | 11.48 | 0.43 | 3.7 | 1.29 | 0.96 | 8.4 | 2.69 |
| Wholemeal bread | 11.45 | 0.75 | 6.6 | 2.11 | 1.88 | 16.4 | 5.26 |
| Wholemeal/white bread (1:1) | 8.53 | 1.03 | 12.0 | 2.87 | 1.13 | 13.2 | 3.15 |
| White bread | 4.19 | 0.64 | 15.2 | 1.78 | 0.73 | 17.4 | 2.04 |
| High bran bread | 11.85 | 1.64 | 13.9 | 4.60 | 2.04 | 17.2 | 5.70 |
| Rye bread | 6.08 | 0.35 | 5.7 | 0.98 | 0.75 | 12.3 | 2.10 |
| Cornflakes | 3.89 | 0.61 | 15.8 | 1.72 | 0.70 | 18.1 | 1.97 |
| Oat cereal | 9.30 | 0.49 | 5.2 | 1.36 | 1.76 | 18.9 | 4.93 |

A6 Key to Tables 1 and 2

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

J. Assoc. Publ. Analysts, 1992, 28, 25-35

# MAFF VALIDATED METHODS FOR THE ANALYSIS OF FOODSTUFFS

**No. V2**

**DIETARY FIBRE (GLC)**

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

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

## 1. Scope and field of Application

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

## 2. Definition

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

## 3. Principle

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

## 4. Reagents

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

**4.1** Acetone

**4.2** Dimethyl sulphoxide

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

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

**4.4.1** Sodium acetate, 0.1 mol/l: prepare by dissolving 13.6 g sodium acetate trihydrate, $CH_3COONa.3H_2O$, and making up to 1 litre with benzoic acid (**4.3**).

**4.4.2** Acetic acid, 0.1 mol/l

**4.4.3** Calcium chloride, 1 mol/l

**4.4.4** Sodium acetate buffer, 0.1 mol/l: adjust sodium acetate, 0.1 mol/l (**4.4.1**) to pH 5.2 with acetic acid, 0.1 mol/l (**4.4.2**). To stabilise and activate enzymes, add 4 ml of calcium chloride, 1 mol/l (**4.4.3**) to 1 litre of buffer.

**4.5** α-Amylase, EC3.2.1.1: Pancrex V Capsules (approximately 9000 BP units α-amylase per capsule, Pains and Byrne Ltd.).

**4.5.1** α-Amylase solution, 2000 BP units/ml: empty 2 Pancrex V Capsules (**4.5**) into a centrifuge tube. Add 9 ml of water and disperse using a vortex-mixer. Centrifuge at 1500 g for 10 min. and use the supernatant as α-amylase solution. Prepare immediately before use.

**4.6** Pullulanase, 100 units/ml: EC 3.2.1.41 (Boehringer 108944).

**4.6.1** Pullulanase solution, 1 unit/ml: dilute pullulanase (**4.6**) 1:100 (e.g. 0.010 ml made up to a final volume of 1 ml) with acetate buffer (**4.4.4**). Prepare immediately before use.

**4.7** Ethanol, absolute.

**4.8** Ethanol, 85% (V/V).

**4.9** Sulphuric acid, 12 mol/l.

**4.10** GLC Internal standard solution, 1 mg/ml: accurately weigh allose (dried to constant weight under reduce pressure with phosphorus pentoxide, $P_2O_5$) and make up the volume with benzoic acid solution (**4.3**).

**4.11** Octan-2-ol

**4.12** Ammonium hydroxide, 12 mol/l.

**4.13** Ammonium hydroxide/sodium borohydride solution: ammonium hydroxide, 3 mol/l, containing 50 mg/ml sodium borohydride ($NaBH_4$).

**4.14** 1-Methylimidazole

**4.15** Acetic acid, glacial.

**4.16** Acetic anhydride

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

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

**4.19** Sulphuric acid, concentrated.

**4.20** Dimethylphenol solution: dissolve 0.1 g of 3,5-dimethylphenol, $(CH_3)_2C_6H_3OH$, in 100 ml of glacial acetic acid (**4.15**).

**4.21** Glucuronic acid, solid: for preparation of standards used in uronic acid determination (dried to constant weight under reduced pressure with phosphorus pentoxide, $P_2O_5$).

**4.22** Standard sugar solutions, 1 mg/ml: use pure preparations of L-rhamnose, L-arabinose, D-xylose, D-mannose, D-galactose and D-glucose (dried to constant weight under reduced pressure with $P_2O_5$). Dissolve the sugars in, and make up the volume with benzoic acid solution(**4.3**).

**4.23** Sulphuric acid, 2 mol/l.

## 5. Apparatus

**5.1** Centrifuge

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

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

**5.4** Hot block

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

**5.6** Vortex-mixer

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

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

**5.9** Oven, capable of maintaining a temperature of 42 ± 2°C for 24 hr.

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

| | | |
|---|---|---|
| **Chromatographic column** | : | **2.1 m x 3 mm i.d., glass** |
| **Column packing** | : | **Supelcoport (100/120 mesh) coated with SP 2330** |
| **Injector temperature** | : | **275°C** |
| **Column temperature** | : | **215°C (isothermal)** |
| **Detector temperature** | : | **275°C** |
| **Carrier gas** | : | **Nitrogen** |
| **Carrier gas flow-rate** | : | **25 ml/min.** |

## 6. Procedure

**6.1** Pre-treatment of sample

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

**6.2** Test samples

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

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

**6.3** Fat extraction

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

**6.4** Dispersion and enzymic hydrolysis

**6.4.1** Dispersion of the starch

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

Remove tube (a) from the beaker and immediately, without cooling, add 8 ml of acetate buffer (**4.4.4**), pre-equilibrated at 50 ± 5°C, and vortex-mix. Then remove tube (b) from the beaker.

**6.4.2** Enzymic hydrolysis of the starch

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

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

Incubate the sample at 42 ± 2°C (**5.7** or **5.9**) for 16 to 18 hrs. Vortex-mix after the first hr.

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

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

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

**6.5** Acid hydrolysis of the residue from enzymic digestion

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

**6.6** Determination of neutral sugars by GLC

**6.6.1** Preparation of alditol acetate derivatives.

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

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

**6.6.2** GLC

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

**6.7** Determination of uronic acids

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

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

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

## 7. COSHH

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

## 8. Expression of Results

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

**8.1** Calculation of neutral sugars

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

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

where:

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

$A_t$ is the peak area for the test solution.

$A_s$ is the peak area for the internal standard.

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

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

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

where:

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

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

where:

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

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

**8.2** Corrections

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

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

**8.3** Calculation of non-starch polysaccharides

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

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

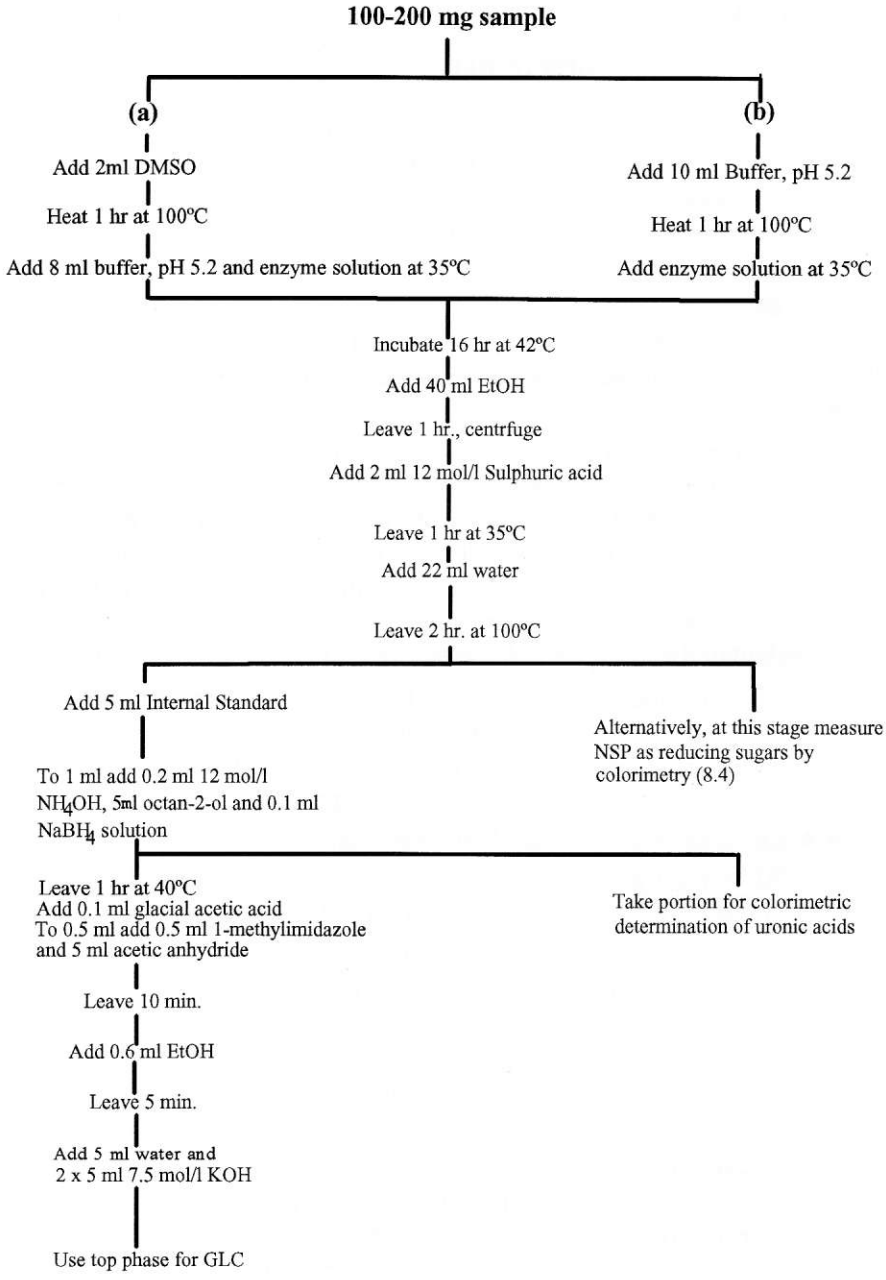
NSP + RS = Rhamnose × 2 + arabinose + xylose + mannose + galactose + glucose, all as measured in section 7.1 for test portion (b) + uronic acids as measured in section 6.7 for test portion (b).

**8.5** Calculation of resistant starch

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

## APPENDIX 1

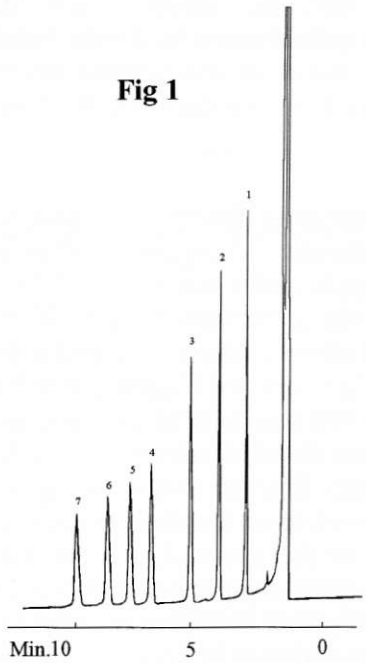
### Schematic Diagram for Analysis of Dietary Fibre



## APPENDIX 2

### Typical Chromatogram of Standard Sugars

**Fig 1**




### Standard sugar solution

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

### GLC column and conditions

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

## 9. References

**9.1** HN Englyst, JH Cummings and R Wood. J. Assoc. Publ. Analysts, 1987, **25**, 73-110.

**9.2** HN Englyst and JH Cummings. Analyst, 1984, **109**, 937-942.

**9.3** HN Englyst and JH Cummings. J. Assoc. Off. Analyt. Chem., 1988, **71**, 808-814.

**9.4** MAFF Validated Methods for the Analysis of Foodstuffs. No.V1; Dietary Fibre, Englyst Method: Colorimetric End-point Determination. London, 1990.

## APPENDIX 3

### Analytical Quality Control

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

## A1 Repeatability

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

## A2 Reproducibility

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

## A3 Trueness (Bias)

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

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

## A4 Limit of Detection

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

## A5 Statistical Data derived from the Results of Interlaboratory Tests

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

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

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

**TABLE 1**

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

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

**TABLE 2**

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

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

**A6 Key to Tables 1 and 2**

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

J. Assoc. Publ. Analysts, 1992, 28, 37-42

# MAFF VALIDATED METHODS FOR THE ANALYSIS OF FOODSTUFFS

## No. V3

## ERUCIC ACID IN OILS AND FATS

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

### 1. Scope and Field of Application

The method allows the determination of the erucic acid (*cis*-13-docosenoic acid) content of oils and fats, including: (i) oils and fats containing cetoleic acid (a *cis*-isomer of docosenoic acid, which occurs in fish oils), and (ii) hydrogenated oils and fats containing *trans*-isomers of docosenoic acid.

### 2. Definition

**Erucic acid content**: the content of erucic acid as determined by the method specified.

### 3. Principle

Separation of the methyl esters of the component fatty acids by low temperature argentation thin-layer chromatography and quantitative determination of the separated esters by gas-liquid chromatography.

### 4. Reagents

All solvents and chemicals should be of analytical reagent quality.

**4.1** Diethyl ether, peroxide-free.
**4.2** *n*-Hexane
**4.3** Silica gel G, for thin-layer chromatography.
**4.4** Silica gel, for column chromatography.
**4.5** Silver nitrate solution, 200 g/l: dissolve 24 g of silver nitrate in water and dilute to 120 ml with water.
**4.6** Methyl erucate solution, 5 mg/ml: dissolve 50 mg of methyl erucate in light petroleum (b.p.40 - 60°C) and dilute to 10 ml.
**4.7** Methyl tetracosanoate (internal standard solution), 0.25 mg/ml: dissolve 25 mg of methyl tetracosanoate in light petroleum (**as 4.6**) and dilute to 100 ml.
**4.8** Development solvent, toluene and hexane diluted 1 litre + 1 Litre.
**4.9** 2,7-Dichlorofluorescein solution, 0.5 g/l: dissolve by warming and stirring 50 mg of 2,7-dichlorofluorescein in 100 ml of 50%(*V/V*) aqueous methanol.

### 5. Apparatus

**5.1** Thin-layer chromatography

The apparatus required for thin-layer chromatography to include, in particular, the following:

**5.1.1** Deep-freeze unit, capable of maintaining the development tank and contents at a temperature of between - 20°C and - 25°C.

**5.1.2** Glass plates, 200 mm x 200 mm.

**5.1.3** Ultra-violet lamp

**5.1.4** Column, glass, length about 200 mm, internal diameter about 10 mm, wit h sintered-glass filters.

**5.1.5** Applicator, for depositing solutions in the form of a narrow band or streak on the plates.

**5.2** Apparatus for gas-liquid chromatography, fitted with an electronic integrator.

## 6. Procedure

**6.1** Preparation of fatty acid methyl esters

Take a representative sample of about 400 mg of the oil or fat and prepare a solution containing about 20-50 mg/ml of the fatty acid methyl esters in hexane.

**6.2** Thin-layer chromatography

**6.2.1** Preparation of plates

Place 60 g of silica gel in a 500 ml round-bottom flask with 120 ml of silver nitrate solution and shake for one minute to obtain a fully homogeneous slurry. Spread the slurry in the usual manner over the plates, adjusting the layer thickness to 0.5 mm. This quantity of slurry is sufficient for the preparation of five 200 ×200 mm plates. Allow the plates partially to air-dry (preferably in the dark for about 30 minutes) and then dry and activate them in an oven at 100°C for 2.5 hr. The plates should be used as soon as possible after activation, otherwise they should be stored carefully in a dark cabinet and activated before use. (Note: activation at 110°C for 1 hr. may be found satisfactory provided the plates are not darkened.) Score lines through the coating 10 mm from the sides and the top of each plate before use to reduce edge effects during the development.

**6.2.2** Application of methyl esters

Using the applicator, deposit 50 µl of the prepared sample methyl esters solution in a narrow streak about 50 mm long, at least 40 mm from the side of the plate and 10 mm from the bottom. Apply in a similar way 100 µl of a solution containing equal volumes of the prepared solution of methyl esters (**6.1**) and the methyl erucate solution (**4.6**). Particular care is necessary during the application of solutions because of the fragile nature of the coating. (Note: if desired, 50 µl of the methyl erucate solution may be applied to the plate as above to assist in identification of the methyl erucate band after development.) After the application of the methyl esters the bottom edge of the plate may be stood in diethyl ether until the ether ascends to about 5 mm above the area of sample application; this will concentrate the methyl esters into a narrow band.

**6.2.3** Development of plates

Pour sufficient development solvent into the tank to give a depth of about 5 mm and place the tank, complete with lid, in a deep freeze cabinet at - 25°C, or as near to this temperature as possible. (Lining the tank with paper may in some cases be advantageous.) After two hr. place the plate carefully in the tank and allow the solvent to ascend to about one-half to two-thirds of the height of the plate. Remove the plate and gently evaporate off the solvent from the plate with a stream of nitrogen. Replace the plate in the tank and allow the solvent to ascend to the top of the plate. Remove the plate and dry with a stream of nitrogen as before, then spray with 2,7-dichlorofluorescein solution. When viewed under ultra-violet light, the methyl erucate in the sample can be located by reference to the intensified band in the sample to which methyl erucate has been added.

**6.2.4** Separation of the methyl ester fractions

Scrape off quantitatively the methyl erucate band derived from the sample into a 50 ml beaker. Scrape off quantitatively the silica gel above and below the methyl erucate band, which contains all the other fatty acid methyl ester fractions, into another 50 ml beaker. To each beaker add 1.0 ml of the methyl tetracosanoate standard solution and 10 ml of diethyl ether. Stir and transfer separately the contents of the beakers to the column or filters containing about 1 g of silica gel, and extract the esters using three or four 10 ml portions of diethyl ether. Collect the filtrates in small flasks. Evaporate the filtrates to small volumes under a gentle stream of nitrogen and transfer them to small glass tubes with pointed bottoms. Remove all solvent by evaporation under nitrogen in such a way that the methyl esters are concentrated at the bottom of the tubes. Dissolve the methyl esters in about 25-50 µl of hexane.

**6.3** Gas-liquid chromatography

Inject 1-2 µl of the solutions of methyl esters obtained from (i) the fraction containing methyl erucate and (ii) the fractions containing the remainder of the fatty acid methyl esters. From the electronic integrator obtain the following peak areas:

(i) from the chromatogram of the fraction containing methyl erucate,

(a) methyl erucate [$E$];

(b) internal standard [$L_1$];

(c) total methyl ester peak areas excluding the internal standard [$EF$];

(ii) from the chromatogram of the fractions containing the remainder of the fatty acid methyl esters,

(a) total peak areas excluding the internal standard [$RF$];

(b) internal standard [$L_2$].

## 7. COSHH

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

## 8. Expression of Results

### 8.1 Formula and method of calculation

The erucic acid content of the sample, expressed as a percentage of the total fatty acids, is given by:

$$\% \text{ erucic acid } = \frac{E}{L_1\{EF/L_1 + RF/L_2\}} \times 100$$

where $E$, $EF$, $RF$, $L_1$ and $L_2$ are the peak areas referred to in the previous section, corrected as necessary by the use of calibration factors. If peak areas are obtained in percentages, the values for $EF$ and $RF$ may be calculated as follows:

$$EF = 100 - L_1$$
$$RF = 100 - L_2$$

The method of calculation assumes that the level of tetracosanoic acid in the sample is negligible. If significant amounts of this acid are found to be present, the value for tetracosanoic acid ($L_2$) obtained from the chromatogram of the fractions containing the remainder of the fatty acid methyl esters must be reduced to $L_2$*, where:

- $L_2$* is the peak area of tetracosanoic acid derived from the internal standard and is calculated using $L_2^* = L_2 - T_2$,
- $T_2$ is the peak area of tetracosanoic acid derived from the sample and which forms part of the peak area attributed to the internal standard in the chromatogram of the remaining fraction of fatty acid methyl esters and is calculated using $T_2 = T_0 P_2 / P_0$,
- $P_2$ is the peak area of palmitic acid obtained from the same chromatogram as $T_2$,
- $T_0$ is the peak area of tetracosanoic acid obtained from the chromatogram of the total fatty acids,
- $P_0$ is the peak area of palmitic acid obtained from the same chromatogram as $T_0$.

### 8.2 Derivation of formula

The fatty acid content of the fraction containing methyl erucate, expressed as a percentage of the total fatty acids in the sample, is given by:

$$\% \text{ total fatty acids } = \frac{EF/L_1}{EF/L_1 + RF/L_2^*} \times 100$$

or

$$\% \text{ total fatty acids } = \frac{EF}{L_1\{EF/L_1 + RF/L_2^*\}} \times 100$$

The erucic acid content of the fraction containing methyl erucate, expressed as a percentage of the fatty acids in the methyl erucate fraction, is given by:

$$\% \text{ erucic acid in methyl erucate fraction} = E/EF \times 100$$

Hence the erucic acid content of the sample, expressed as a percentage of the total fatty acids, is given by:

$$\% \text{ erucic acid content} = \frac{EF}{L_1\{EF/L_1 + RF/L_2^*\}} \times \frac{E}{EF} \times 100$$

or

$$\% \text{ erucic acid content} = \frac{E}{L_1\{EF/L_1 + RF/L_2^*\}} \times 100$$

## 9. References

**9.1** RB Player and R Wood, J. Assoc. Publ. Analysts, 1980, **18**, 77- 89

**9.2** RS Kirk, RE Mortlock, WD Pocklington and P Roper, J. Sci. Fd. Agric., 1978, **29**, 880.

## APPENDIX 1

### Analytical Quality Control

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

### A1 Repeatability

The absolute difference between two test results obtained under repeatability conditions should not be greater than the repeatability, r, deduced from the collaborative trial data summarised in Table 1. At about 7% erucic acid, r may be taken as 1.3%; at 11% erucic acid r is about 1.9%. This precision corresponds to an overall relative standard deviation of repeatability (coefficient of variance of repeatability), $RSD_r$, of about 7%.

### A2 Reproducibility

The absolute difference between two test results obtained under reproducibility conditions should not be greater than the reproducibility, R, deduced from the collaborative trial data summarised in Table 1. R may be taken as 4% at erucic acid levels of 7%; this precision corresponds to a relative standard deviation of reproducibility (coefficient of variance of reproducibility), $RSD_R$, of some 20%.

### A3 Trueness (Bias)

The collaborative trial included the analysis of a sample of oil before (A) and after (D) spiking with 4.78% authentic methyl erucate. An overall mean difference of 4.37% erucic acid was found, suggesting a recovery of 95%; however there was no statistical evidence that this recovery was less than quantitative. The method is therefore unlikely to be systematically biased.

## A4 Limit of Detection

This limit has not been established, but the collaborative trial data suggests an accuracy which, if maintained, corresponds to an extrapolated lower limit of roughly 1% erucic acid for a single determination.

## A5 Statistical Data Derived from the Results of Interlaboratory Tests

Participants in the collaborative trial each analysed four samples (A-D) in duplicate. The samples were known mixtures of rapeseed oil, corn oil and/or partially hydrogenated marine oil; one of them (D) was sample A spiked with 5% methyl erucate. A summary of statistical data is tabulated below; the erucic acid levels were expressed as a percentage of total fatty acids.

**TABLE 1**

**Statistical Analysis of the % Erucic Acid in Mixed Oil Samples**

| Sample | A | B | C | D |
|---|---|---|---|---|
| Number of Laboratories retained after eliminating outliers | 18 | 18 | 18 | 17 |
| Number of Laboratories eliminated as outliers | 6 | 6 | 5 | 7 |
| Number of results accepted after eliminating outliers | 33 | 33 | 35 | 32 |
| LEVEL OF ANALYTE | | | | |
| Mean observed value $\bar{x}$ | 6.84 | 6.08 | 6.10 | 11.21 |
| REPEATABILITY | | | | |
| Standard Deviation $S_r$ | 0.41 | 0.47 | 0.46 | 0.69 |
| Relative Standard Deviation $RSD_r$ (%) | 6.0 | 7.7 | 7.5 | 6.2 |
| Repeatability r [2.5 x $S_r$] | 1.16 | 1.32 | 1.29 | 1.94 |
| REPRODUCIBILITY | | | | |
| Standard Deviation $S_R$ | 1.43 | 1.25 | 1.21 | 1.57 |
| Relative Standard Deviation $RSD_R$ (%) | 20.9 | 20.6 | 19.8 | 14.0 |
| Reproducibility R [2.5 x $S_R$] | 4.00 | 3.51 | 3.39 | 4.40 |

## A6 Key to Table 1

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

J.Assoc.Publ.Analysts, 1992, 28, 43-49

# MAFF VALIDATED METHODS FOR THE ANALYSIS OF FOODSTUFFS

## No.V4

## BIPHENYL AND 2-HYDROXYBIPHENYL IN CITRUS FRUITS

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

### 1. Scope and Field of Application

The method allows the examination of citrus fruits and their juices for trace residues of the fungicides biphenyl and 2-hydroxybiphenyl; it determines both analytes at levels corresponding to statutory limits (70 mg/kg and 12 mg/kg respectively) and will detect levels down to 1 mg/kg.

### 2. Definition

**Biphenyl content**: the content of biphenyl as determined by the method specified.

**2-Hydroxybiphenyl content**: the content of 2-hydroxybiphenyl as determined by the method specified.

### 3. Principle

Steam distillation using a modified Clevenger separator, extraction into organic solvent and quantitative gas-liquid chromatography.

### 4. Reagents

**4.1** Anti-foaming Tablets

**4.2** Anti-bumping granules

**4.3** Sulphuric acid, 50% (*V/V*).

**4.4** Cyclohexane, spectrosol grade.

**4.5** Sodium sulphate, anhydrous

**4.6** Heptadecane, 10 mg/ml: dissolve 500 mg AR grade heptadecane in cyclohexane and make up to 50 ml in a volumetric flask.

**4.7** 2-Hydroxybiphenyl, 10 mg/ml: dissolve 500 mg AR grade 2-hydroxybiphenyl in cyclohexane and make up to 50 ml in volumetric flask.

**4.8** Biphenyl, 10 mg/ml: dissolve 500 mg AR grade biphenyl in cyclohexane and make up to 50 ml in a volumetric flask.

### 5. Apparatus

**5.1** Food-mixer, Kenwood, or similar with liquidiser attachment.

**5.2** Coffee grinder

0004-5780/93 +7 $20.00

**5.3** Distillation apparatus: 1 litre round-bottom flask fitted with a modified Clevenger separator (see Fig. 1) and a water jacket condenser.

**5.4** Heating mantle

**5.5** Vacuum evaporator with 50 ml flasks.

**5.6** Gas chromatograph

Becker model 420 fitted with a flame ionisation detector and connected to a Servoscribe chart recorder, model RE541. Column: glass, 2 m x 4.0 mm i.d., packed with 3% OV-17 on Gas Chrom. Q. Injector temperature: 210°C. Column temperature-programme: 130°C for 10 min., 130-160°C at 10°C/min, 160°C for 22 min. Detector temperature: 225°C. Carrier gas flow rate: 30 ml nitrogen per min. Detector gas pressures: hydrogen and air set according to manufacturer's instructions.

## 6. Procedure

**6.1** Preparation of Extracts

Separate the flesh and the peel from at least 2 kg of fruit. Cut the peel into small pieces and grind in a coffee grinder. Homogenise the flesh in a liquidiser. Combine the portions and mix well. Take 300 g of recombined fruit pulp and add 300 ml of water. Homogenise at low speed in a mixer and pour into the 1 litre round-bottom flask, rinsing the homogeniser with 50 ml water and adding this to the flask. Add an anti-foam tablet, a few anti-bumping granules and 15 ml of 50% sulphuric acid. Assemble the distillation apparatus and place 15 ml of water and 20 ml of cyclohexane in the side arm of the separator. Boil the mixture gently for 20 min. and then more vigorously for the remainder of 2 hr. such that one drop distils every 3-5 sec. At the end of this time allow the mixture to cool.

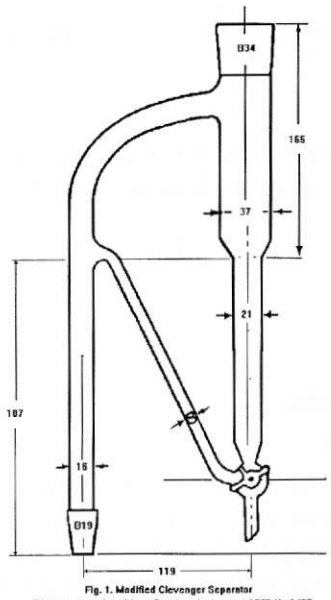


Fig. 1. Modified Clevenger Separator
Diagram reproduced from Statutory Instrument 1975 No.1487
Dimensions in millimetres

Run the water from the Clevenger separator into a 100 ml separating funnel and the cyclohexane into a 25 ml volumetric flask. Rinse the Clevenger separator with 4 ml of cyclohexane and add this to the water in

the separating funnel. Shake well, allow the layers to separate, discard the water and add the cyclohexane to that in the 25 ml volumetric flask. Make up to volume with cyclohexane, add a little anhydrous sodium sulphate and mix well (this is extract "a"). Set up the separator again with a fresh portion of 20 ml of cyclohexane and repeat the two-hour distillation and subsequent separation as above (this is extract "b").

**6.2** Preparation of standard graphs

Prepare five standard solutions (A-E) containing the indicated amounts of fungicide and internal standard.

| | A | B | C | D | E |
|---|---|---|---|---|---|
| Volume (ml) of biphenyl solution 10mg/ml | 0.4 | 0.8 | 1.2 | 1.6 | 2.0 |
| Volume (ml) of 2-hydroxybiphenyl 10mg/ml | 0.1 | 0.2 | 0.3 | 0.4 | 0.5 |
| Volume (ml) of heptadecane 10mg/ml | 1.0 | 1.0 | 1.0 | 1.0 | 1.0 |

Make each up to 5 ml with cyclohexane.

Inject 1.5 µl of each standard and record the chromatogram. Measure the areas (height x width at half height) of the peaks. Plot the area ratio of fungicide to internal standard against mg fungicide per 10 mg of heptadecane for each compound.

**6.3** Determination of biphenyl

To 10 ml of extract "a" add 0.4 ml of heptadecane solution, 10 mg/ml. Inject 1.5 µl and record the chromatogram. Measure the peak areas and calculate the ratio of peak area of heptadecane. From the standard graph read off the biphenyl content, allowing for the amount of heptadecane added.

If necessary the solution may be reduced in volume under vacuum but because of the volatility of biphenyl the volume should not be taken below 2 ml. A typical chromatogram is shown in fig. 2A.

**6.4** Determination of 2-hydroxybiphenyl

Combine 10ml of extract "a" and 10ml of extract "b" and add 0.1 ml of heptadecane solution, 10 mg/ml. Evaporate the solution under vacuum but without rotating the flask until the volume is reduced to a few ml. Inject 1.5 µl and record the chromatogram. Measure the peak areas and calculate the ratio of peak area of 2-hydroxybiphenyl to peak area of heptadecane. From the standard graph read off the 2-hydroxybiphenyl content, allowing for the amount of heptadecane added. The attenuation of the instrument should be adjusted so that the heptadecane peak is at least 40% of maximum. The biphenyl peak may then be off scale.

Care should be taken to identify correctly the 2-hydroxybiphenyl peak particularly if it is small; natural substances present in citrus fruit sometimes give peaks corresponding to 0.5-1.0 mg/kg of 2-hydroxybiphenyl at the same retention time. A typical chromatogram is shown in fig. 2B.

**Fig. 2A**

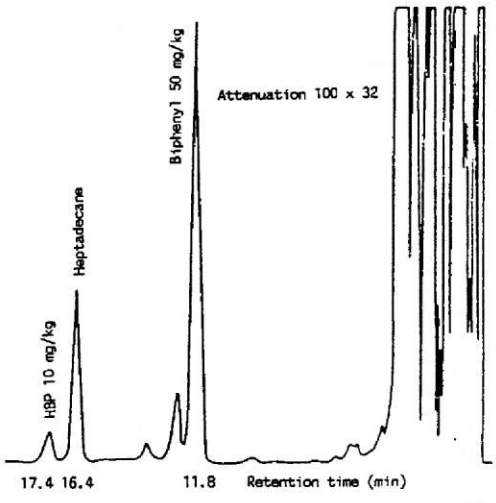



*Fig.2a Chromatogram for the determination of: biphenyl*

**Fig. 2B**

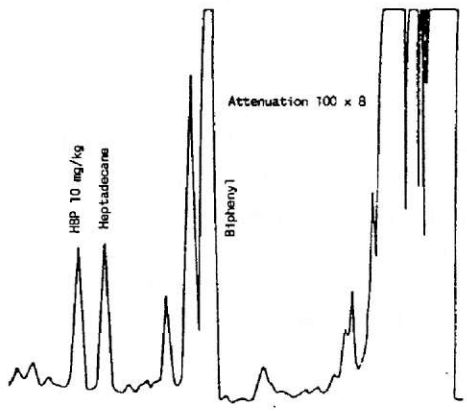



*Fig.2b Chromatograms for the determination of 2-hydroxybiphenyl*

Two 300 g portions from the same sample of oranges spiked with 15 mg biphenyl (50 mg/kg) and 3 mg 2-hydroxybiphenyl (10 mg/kg) were treated by the distillation, extraction and glc procedures described in the text.

## 7. COSHH

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

## 8. Expression of Results

Calculate the levels of biphenyl and 2-hydroxybiphenyl and express as mg/kg sample.

## 9. References

**9.1** E Lord, NG Bunton and NT Crosby, J. Assoc. Publ. Analysts, 1978, **16**, 25-32.

**9.2** RB Player and R Wood, J. Assoc. Publ. Analysts, 1980, **18**, 109-117.

## APPENDIX 1
## Analytical Quality Control

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

### A1 Repeatability

The absolute difference between two test results obtained under repeatability conditions should not be greater than the repeatability, r, deduced from the collaborative trial data summarised below (Table 1). When biphenyl is the analyte at levels of about 85 mg/kg, r may be taken as 8.9 mg/kg; with 2-hydroxybiphenyl at about 15 mg/kg, the corresponding value of r is 1.6 mg/kg.

This precision corresponds to a relative standard deviations of repeatability (Coefficient of variance of repeatability), $RSD_r$, of about 5.4% and 7.9% respectively.

### A2 Reproducibility

The absolute difference between two test results obtained under reproducibility conditions should not be greater than the reproducibility, R, deduced from the collaborative trial data summarised below (Paragraph 6, Table 1) in the same way as repeatability above. The recommended values of R are 21 mg/kg at 85 mg biphenyl/kg and 9 mg/kg at 15 mg 2-hydroxybiphenyl/kg. This precision corresponds to a relative standard deviations of reproducibility (Coefficient of variance of reproducibility), $RSD_R$, of about 13% and 40% respectively. It should be noted that close quantitative agreement between laboratories analysing

very low levels of 2-hydroxybiphenyl cannot be expected, but the method is still recommended until improvements can be demonstrated.

**A3 Trueness (Bias)**

The collaborative trial was based on the analysis of a sample of comminuted orange (including juice) before and after spiking with 84 mg biphenyl/kg, 14.4 mg 2-hydroxybiphenyl/kg and 12.0 mg thiabendazole/kg. The untreated sample was shown to contain about 1 mg of each analyte/kg. The results summarised in paragraph 5 demonstrated that only 70% of the biphenyl and 48% of the 2-hydroxybiphenyl were recovered. While these recoveries are lower than would be normally regarded as satisfactory, they are acceptable for general control purposes, since alternative methods, requiring 50% more time to undertake, have similar recoveries.

**A4 Limit of Detection**

This limit has not been established. However, the analysis of the unspiked sample during the collaborative trial indicated that these control levels were at or close to the limit of detection. The true levels were unknown; the observed levels were subject to considerable error, but indicated a limit of detection of roughly 1 mg of either analyte/kg.

**A5 Statistical Data Derived from the Results of Interlaboratory Tests**

Twenty-two laboratories agreed to analyse two samples of a natural comminuted orange product, one of which had been spiked with known levels of biphenyl, 2-hydroxybiphenyl and thiabendazole. While other methods were also tested, the data summarised in Table 1 result from the application of the method described in this booklet to the spiked sample only. The results of one laboratory were omitted because the prescribed method was not followed. The levels of each analyte were expressed as mg/kg sample.

**TABLE 1**

**Statistical Analysis of Biphenyl and 2-Hydroxybiphenyl in Natural Comminuted Orange Product Samples**

| Sample | biphenyl | 2-hydroxybiphenyl |
|---|---|---|
| Number of laboratories retained after eliminating outliers | 21 | 21 |
| Number of laboratories eliminated as outliers | 0 | 0 |
| Number of results accepted | 42 | 40 |
| LEVEL OF ANALYTE | | |
| Mean observed value $\bar{x}$ | 59.6 | 7.33 |
| Accepted "true" value | 85 | 15.4 |
| REPEATABILITY | | |
| Standard Deviation $S_r$ | 3.19 | 0.58 |
| Relative Standard Deviation $RSD_r$(%) | 5.4 | 7.9 |
| Repeatability r [2.8 x $S_r$ ] | 8.94 | 1.62 |
| REPRODUCIBILITY | | |
| Standard Deviation $S_R$ | 7.46 | 2.91 |
| Relative Standard Deviation $RSD_R$(%) | 12.5 | 39.7 |
| Reproducibility R [2.8 x $S_R$ ] | 20.9 | 8.16 |

## A6 Key to Table 1

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