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# Determination of Papain in Raw Meat Collaborative Trial

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*Eighteen laboratories participated in a collaborative trial to evaluate an immunological method for the determination of papain in raw meat. The method tested was shown to be accurate and exhibited acceptable precision characteristics, and so can be recommended for use for the determination of papain in such samples.*

Tenderness is one of the most important qualities of meat, yet it is a quality that is subject to a wide variation not only from one animal to another but also between cuts of meat from the same animal. This variation is attributable to qualitative and quantitative aspects of connective tissue in the muscles of the animal.

In recent years it has been possible to artificially tenderise meat by using proteolytic enzymes which can be introduced into the animal pre-slaughter by injection into the jugular vein. This technique ensures a reasonable distribution of the enzyme into the muscle tissues. Papain is the proteolytic enzyme most commonly used for meat tenderisation and it is used at a level that results in a concentration of up to 1 mg/kg being found in the muscle tissues. It acts preferentially upon connective tissue fibres[(1)]. In this respect, the mucopolysaccharide of the ground substance matrix is degraded first followed by disintegration of the connective tissue fibres to an amorphous mass and the release of soluble hydroxyproline containing molecules. These enzymatic changes occur only during cooking of the meat when heat denaturation of connective tissue commences, and the enzyme approaches its temperature optimum.

The use of such tenderisers is permitted provided that the food is correctly labelled.

A procedure has been developed to enable to determination of papain in raw meat and thus ascertain whether a sample has been tenderised. It is based on an immunological method for the quantitative determination of papain in raw meat; the development of that procedure has been described elsewhere[(2)].

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## Method of Analysis

### Development

The method used in the collaborative trial is based on the immunological procedure described previously[3].

### Kit

For this trial the method reagents etc. were prepared in "kit" form and then distributed to participants. The development of the kit required that the materials for the assay should be presented as stable reagents, ready for use where possible; the rationale being that this would minimise variation associated with make-up of reagents by individual laboratories.

In this respect the kit components were presented as follows:

a) Microwells were pre-coated with affinity purified rabbit anti-papain antibody, dried and packed in moisture-proof foil pouches with desiccant.

b) Calibration standard meat extracts were prepared in bulk from a spiked beef sample known previously to be free of papain. These standard extracts (0 - 1 mg/kg papain) were treated with preservative and were found to be stable for at least 3 months at 4°C. As a precaution the participants were asked to store them frozen during the trial.

c) Horseradish peroxidase conjugated rabbit anti-papain IgG was prepared in phosphate buffered saline pH 7.2 with 0.2% BSA and 0.01% thiomersal as preservative. The conjugate was supplied as a working solution ready for use.

d) The ABTS substrate (0.5 mg/ml) was presented in citrate-phosphate buffer containing $H_2O_2$, as a one-component substrate system ready to use.

The stability of reagents used in the trial was such that no detectable changes could be observed within a 3 months storage period at 4°C.

## Collaborative Trial Organisation, Samples and Results

### Laboratories

Eighteen UK laboratories participated in the trial, only one of which had had any direct experience with immunological papain assays before participating in this trial.

### Participants

G Baker, M Barnett, M Billington, W Cassidy, B Dredge, D Dunn, A Ellis, J Fulstow, S Guffogg, A J Harrison, G Hooke, T E Johnson, G Keen, I Lumley, R Nicolson, E B Reynolds, B Sanders, and B Taylor

### Method

For this collaborative trial the method to be used is as given in Appendix I. However, each participant was supplied with reagents in "kit" form, as also described in Appendix I.

## Samples

For this collaborative trial each participant was supplied with 1 Papain Immunoassay Kit and 12 chilled homogenised beef samples. Of these samples, 2 (labelled pre-trial 1 & 2), were for use in the pre-trial, and were to be analysed as known duplicates, and 10 samples (labelled A-J) were for use in the trial proper, and were to be analysed once only. These ten samples were prepared from five base samples and distributed as blind duplicates.

## Sample Preparation

The test beef samples supplied to each participant were prepared by the exogenous addition of papain to minced beef. The methods involved in sample preparation are given below:

Stewing beef (3.5 kg) was purchased and assayed to ensure that it was free of papain. Portions (500 g) were chopped by hand into 1 cm cubes and reduced to a course mince in a Braun food processor.

Papain stock solution was prepared by dissolving papain (2X crystallised and lyophilised - Sigma P 4762) in phosphate buffered saline (PBS) to give a stock solution of 1 mg/ml). This stock solution was diluted to give the concentrations described in Table I.

**TABLE I**

Volume of stock solution used in preparation of samples

| SAMPLE | Vol stock soln. in 10 ml PBS added to 500 g beef | Papain Conc. mg/kg |
|---|---|---|
| Pre-trial 1 | 300 µl | 0.60 |
| Pre-trial 2 | 200 µl | 0.40 |
| A&C | 125 µl | 0.25 |
| B&H | 40 µl | 0.08 |
| D&G | 410 µl | 0.82 |
| E&J | 0 | 0.00 |
| F&I | 240 µl | 0.48 |

With the machine on slow speed, 10 ml of papain solution (as described in Table I) was added dropwise to ensure adequate dispersal of papain throughout the mince. The sample was allowed to stand for 1 hour at 4°C and then reduced further to a fine mince/paste with the food processor operating on high speed for 3 minutes. Each batch was subdivided by weighing 10 g portions into disposable 160 ml screw-capped containers (50 samples), and these were stored frozen.

## Homogeneity of Samples

Table II shows the results of the analyses carried out using the immunoassay kit, on each batch of beef prepared for the trial.

**TABLE II**

Homogeneity of Prepared Samples

| SAMPLE | PAPAIN mg/kg by Immunoassay | | | | | |
|---|---|---|---|---|---|---|
| mg/kg Papain | 1 | 2 | 3 | 4 | 5 | MEAN |
| P-T 1 (0.60) | 0.62 | 0.60 | 0.63 | 0.59 | 0.59 | 0.606 |
| P-T 2 (0.40) | 0.39 | 0.38 | 0.39 | 0.39 | 0.38 | 0.386 |
| A&C (0.25) | 0.23 | 0.24 | 0.25 | 0.24 | 0.25 | 0.242 |
| B&H (0.08) | 0.09 | 0.08 | 0.08 | 0.08 | 0.09 | 0.084 |
| D&G (0.82) | 0.79 | 0.82 | 0.80 | 0.84 | 0.80 | 0.810 |
| E&J (0.00) | 0.00 | 0.00 | 0.00 | 0.00 | 0.00 | 0 |
| F&I (0.48) | 0.51 | 0.49 | 0.50 | 0.48 | 0.50 | 0.496 |

P_T: Pre-trail. Figures in brackets donate actual levels of papain in samples,

The method described above for the preparation of test samples was found to be the method that gave the best results in terms of sample homogeneity. The results in Table II show the variability to be expected between samples from the same batch.

The samples used in the trial proper comprised a zero and four different levels of papain.

Five samples from each batch were taken at random and assayed in duplicate for papain before the samples were distributed to ensure that sample homogeneity had been obtained.

Distribution of samples

The two pre-trial samples and ten trial samples (packed in ice) together with the papain immunoassay kit were sent to the participants by overnight carrier.

The beef samples were to be extracted and assayed once (in duplicate) in accordance with the immunoassay protocol. The concentration of papain in the pre-trial 1 sample was 0.60 mg/kg and this was made known to each of the participants. The pre-trial assay involved determining the concentration of papain in both pre-trial samples and reporting the results before proceeding with the trial proper. It was considered that this would allow participants to familiarise themselves with the immunoassay and also give an initial indication of any potential problems. The participants were asked to complete the pre-trial within two weeks of receiving the samples and a further two weeks to complete the main trial.

## Results

The results obtained by the participants are given in Tables III to VIII.

**TABLE III**

Sample 1: 0.60 mg/kg Sample 2: 0.40 mg/kg

| LABORATORY | SAMPLE NUMBER 1 | 2 |
|---|---|---|
| 1 | 0.52 | 0.32 |
| 2 | 0.61 | 0.39 |
| 3 | 0.56 | 0.46 |
| 4 | 0.53 | 0.32 |
| 5 | 0.58 | 0.27 |
| 6 | 0.59 | 0.44 |
| 7 | 0.62 | 0.39 |
| 8 | 0.58 | 0.34 |
| 9 | 0.68 | 0.37 |
| 10 | 0.59 | 0.40 |
| 11 | 0.62 | 0.38 |
| 12 | 0.63 | 0.37 |
| 13 | 0.75 | 0.42 |
| 14 | 0.51 | 0.33 |
| 15 | 0.61 | 0.37 |
| 16 | 0.55 | 0.34 |
| 17 | 0.59 | 0.35 |
| 18 | 0.55 | 0.29 |
| MEAN | 0.593 | 0.364 |
| SD | 0.058 | 0.0496 |
| RSD% | 9.765 | 13.631 |

For Key to Tables III - VIII See Table IX

**TABLE IV**

**Papain concentration from sample preparation is 0.00 mg/kg**

| LABORATORY | SAMPLE LETTER E | J |
|---|---|---|
| 1 | <0.05 | <0.05 |
| 2 | NIL | NIL |
| 3 | <0.05 | <0.05 |
| 4 | 0.00 | 0.00 |
| 5 | NIL | NIL |
| 6 | 0.00 | 0.00 |
| 7 | <0.05 | <0.05 |
| 8 | 0.00 | 0.00 |
| 9 | 0.00 | 0.00 |
| 10 | 0.00 | 0.00 |
| 11 | 0.00 | 0.00 |
| 12 | <0.05 | <0.05 |
| 13 | 0.00 | 0.00 |
| 14 | ND | ND |
| 15 | 0.00 | 0.00 |
| 16 | NIL | NIL |
| 17 | 0.00 | 0.00 |
| 18 | <0.05 | <0.05 |

**TABLE V**

**Papain concentration from sample preparation is 0.08 mg/kg**

| LABORATORY | SAMPLE LETTER B | H |
|---|---|---|
| 1 | 0.11 | 0.08 |
| 2 | 0.09 | 0.07 |
| 3 | 0.11 | 0.09 |
| 4 | 0.07 | 0.08 |
| 5 | 0.07(a) | LOST |
| 6 | 0.08 | 0.08 |
| 7 | 0.08 | 0.07 |
| 8 | 0.07 | 0.07 |
| 9 | 0.10 | 0.09 |
| 10 | 0.10 | 0.10 |
| 11 | 0.09 | 0.08 |
| 12 | 0.07 | 0.07 |
| 13 | 0.11 | 0.07 |
| 14 | 0.08 | 0.07 |
| 15 | 0.09 | 0.08 |
| 16 | 0.12 | 0.10 |
| 17 | 0.09 | 0.08 |
| 18 | 0.09 | 0.08 |
| MEAN | 0.086 | |
| r | 0.032 | |
| $SD_r$ | 0.012 | |
| $RSD_r$ % | 13.441 | |
| R | 0.039 | |
| $SD_R$ | 0.014 | |
| $RSD_R$ % | 16.402 | |

**TABLE VI**

**Papain concentration from sample preparation is 0.25 mg/kg**

| LABORATORY | SAMPLE LETTER A | C |
|---|---|---|
| 1 | 0.24 | 0.25 |
| 2 | 0.23 | 0.21 |
| 3 | 0.29 | 0.27 |
| 4 | 0.24 | 0.20 |
| 5 | 0.26 | 0.24 |
| 6 | 0.24 | 0.22 |
| 7 | 0.23 | 0.25 |
| 8 | 0.21 | 0.23 |
| 9 | 0.30 | 0.30 |
| 10 | 0.28 | 0.29 |
| 11 | 0.26 | 0.30 |
| 12 | 0.21 | 0.29 |
| 13 | 0.30 | 0.20 |
| 14 | 0.25 | 0.26 |
| 15 | 0.26 | 0.26 |
| 16 | 0.30 | 0.33 |
| 17 | 0.25 | 0.25 |
| 18 | 0.24 | 0.23 |
| MEAN | 0.255 | |
| r | 0.071 | |
| $SD_r$ | 0.025 | |
| $RSD_r$ % | 9.988 | |
| R | 0.092 | |
| $SD_R$ | 0.033 | |
| $RSD_R$ % | 12.924 | |

**TABLE VII**

**Papain concentration from sample preparation is 0.48 mg/kg**

| LABORATORY | SAMPLE LETTER F | SAMPLE LETTER I |
|---|---|---|
| 1 | 0.50 | 0.50 |
| 2 | 0.47 | 0.44 |
| 3 | 0.58 | 0.55 |
| 4 | 0.45 | 0.48 |
| 5 | 0.50 | 0.56 |
| 6 | 0.54 | 0.55 |
| 7 | 0.40 | 0.36 |
| 8 | 0.49 | 0.45 |
| 9 | 0.73 | 0.70 |
| 10 | 0.56 | 0.58 |
| 11 | 0.52 | 0.52 |
| 12 | 0.53 | 0.47 |
| 13 | 0.52 | 0.42 |
| 14 | 0.46 | 0.45 |
| 15 | 0.52 | 0.54 |
| 16 | 0.70 | 0.72 |
| 17 | 0.53 | 0.49 |
| 18 | 0.51 | 0.50 |
| MEAN | 0.522 | |
| r | 0.077 | |
| $SD_r$ | 0.027 | |
| $RSD_r$ % | 5.257 | |
| R | 0.24 | |
| $SD_R$ | 0.095 | |
| $RSD_R$ % | 16.297 | |

**TABLE VIII**

**Papain concentration from sample preparation is 0.82 mg/kg**

| LABORATORY | SAMPLE LETTER D | SAMPLE LETTER G |
|---|---|---|
| 1 | 0.84 | 0.76 |
| 2 | 0.69 | 0.75 |
| 3 | >1 (b) | >1 (b) |
| 4 | 0.66 | 0.72 |
| 5 | 0.61 | 0.70 |
| 6 | 0.87 | 0.86 |
| 7 | 0.79 | 0.69 |
| 8 | 0.64 | 0.65 |
| 9 | 1.00 | 1.02 |
| 10 | 0.88 | 0.94 |
| 11 | 0.82 | 0.84 |
| 12 | 1.00 | 0.83 |
| 13 | 0.80 | 0.68 |
| 14 | 0.71 | 0.72 |
| 15 | 0.86 | 0.81 |
| 16 | 1.00 | 1.05 |
| 17 | 0.87 | 0.86 |
| 18 | 0.92 | 0.82 |
| MEAN | 0.814 | |
| r | 0.15 | |
| $SD_r$ | 0.053 | |
| $RSD_r$ % | 6.491 | |
| R | 0.34 | |
| $SD_R$ | 0.12 | |
| $RSD_R$ % | 14.824 | |

**Statistical Analysis of Trial Results**

The results were analysed for outliers by the Cochran's and Grubbs Tests using procedures agreed in the "Protocol for the Design, Conduct and Interpretation of Collaborative Studies", prepared by the IUPAC Interdivisional Working Party for the Harmonisation of Quality Assurance Schemes for Analytical Laboratories[2]. In this trial no outlying results were identified.

**Discussion**

The collaborative trial was completed within the time scale specified, (4 weeks).

The results from the pre-trial indicated that participants were performing the analysis satisfactorily, and so were advised to proceed to the analysis of the trial samples proper.

For the blank samples participants reported 0.00, <0.05, Nil or ND (not detected). In all cases these results were a reflection of a measured absorbance of less than 0.03 absorbance units.

Generally, there were no serious problems encountered during the trial and participants found the immunoassay easy to perform. However, one participant was unable to report a result for sample H, due to loss of sample at some stage in the assay and another was unable to report results for samples E and J as he had difficulty keeping the assay on scale, his highest concentration standard giving an absorbance greater than 2 units.

The mean values obtained by the participants are, for all samples, very close to the added amounts used in the sample preparation. The precision values obtained are satisfactory, being in the range normally expected for the determination of analytes at these concentrations. Values for $RSD_R$ have been predicted by Horwitz from experimental data for a large number of collaborative trials[5]. He predicts that at the concentration ranges of 0.1 and 1 mg/kg the values of $RSD_R$ should be 23 and 16% respectively. In this trial, the values are better than the predicted values.

Although the protocol stated that chromophore development was temperature dependant and a substrate incubation time of 12.5 minutes at 20°C was required to achieve a final absorbance of 1.2-1.4 units, a few laboratories did not appear to make allowances for the obviously elevated temperature within their laboratory and consequently observed absorbance values of 1.5-1.8 units. However, with the exception of the above mentioned laboratories, this did not affect their final results.

With hindsight, it would have been a good idea to include in the immunoassay kit a vial containing developed substrate solution of 1.3 absorbance units, so that participants could have used it as a comparative solution to indicate when to stop the substrate reaction. Also it would have been useful as a check on the correct operation of ELISA plate readers. Two laboratories who had problems initially with the assay found that the fault lay with their plate reader.

## Conclusions

A method for the determination of papain in raw meat sample has been developed and collaboratively tested in its "kit" form. The method has shown to be accurate by comparison between the mean value as determined by the participants and the added papain concentration. The precision of the assay is acceptable, and well within the values which may be predicted from the Horwitz equation.

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B Taylor, County Laboratory, Manchester

also to Dr D Thomas, who carried out the statistical analysis of the results.

## References

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## APPENDIX I
## Method for the Determination of Papain in Raw Meat by Immunoassay

### 1. Scope and Field of Application

The method allows the determinations of papain in raw meat.

### 2. Definition

**Papain content:** the content of papain as determined by the method specified.

### 3. Principle

Essentially, specific antibody attached to the solid phase act as a capture antibody. Standards (within the range 0 - 1 mg/kg) and test extract solutions are then incubated with this antibody-solid phase and any papain present is captured. Enzyme-antibody conjugate, (horseradish peroxidase labelled specific antibody) is then incubated with the solid phase and finally the enzyme substrate is added which produces a chromophore, the intensity of which is proportional to the amount of papain present in the test solution.

The Double Antibody Sandwich ELISA system employed is shown diagrammatically in Figure 1.

### 4. Reagents

**4.1** Kit Components

**4.1.1** Standard Extracts

Six vials containing 2 ml standards supplied for use in the assay as calibration standard meat extracts. They represent the following papain concentrations:-

0, 0.05, 0.1, 0.25, 0.5, 1.0, mg/kg papain.

**4.1.2** Antibody Coated Microwells

Foil laminated bag containing six 2 × 8 stripwells (total 96 wells) each precoated with papain antibody and held in a plastic frame and a desiccant bag.

**4.1.3** Wash Solution Concentrate

One bottle containing 50 ml of wash solution concentrate comprising a 20 fold concentrate of Tris buffered saline with 0.05% Tween 20 and 0.01% thiomersal.

**4.1.4** Conjugate

One vial containing 25 ml anti-papain antibody-enzyme conjugate, ready for use.

**4.1.5** Substrate

One vial containing ABTS substrate in citrate-phosphate buffer containing $H_20_2$, ready for use.

**4.1.6** Stop Solution

One vial containing 9 ml of citric acid stop solution, ready for use.

**4.1.7** Calculations

Three sheets of pre-labelled graph paper/work sheet.

**4.2** Sample Preparation

Phosphate buffered saline (PBS) is required for extraction of meat samples, (90 ml/sample). A suitable PBS solution can be prepared as follows:-

| | |
|---|---|
| Sodium chloride | 16.0 g |
| di-Sodium hydrogen orthophosphate (anhydrous) | 2.3 g |
| (heat in a little distilled $H_20$ to dissolve) | |
| Monobasic potassium phosphate (anhydrous $KH_2PO_4$) | 0.4 g |
| Potassium chloride | 0.4 g |

Dissolve in 2 litres of distilled water.

The pH of this solution is pH 7.2 - 7.4.

## 5. Apparatus

General laboratory glassware and:

**5.1** Pipette, 50-200 µl (Gilson)

**5.2** Pipette, 100-1000 µl (Gilson)

**5.3** ELISA plate reader, (Dynatech)

**5.4** ELISA well washer, (Dynatech) - useful but not essential

**5.5** Multi-channel pipette, 50-200 µl - not essential

## 6. Procedure

**6.1** Schematic Representation of Procedure

A schematic representation of the steps involved in the determination is given below:

| Time | Procedure | Volume | Description |
|---|---|---|---|
| 5 minutes | addition | 200 µl | pippette standard extracts and samples into appropriate wells. |
| 1 hour | incubation | | Incubate at room temperature |
| 5 minutes | wash | | Wash 6 times with working wash solution |
| 5 minutes | addition | 200 µl | pipette conjugate solution |
| 1 hour | incubation | | incubate at room temperature |
| 5 minutes | wash | | wash 6 times with working wash solution |
| 1 minute | addition | 200 µl | pipette substrate solution |
| 10 minutes | incubation | | incubate for 10-12 minutes until top standard reaches 1.2 absorbance units, swirl plate every 2 minutes |
| 1 minute | addition | 50 µl | pipette stop solution and swirl plate to mix |
| 5 minutes | read plate | | Read absorbance on plate reader at 410-420 nm |

Total assay time is about 2.5 hours

**6.2** Preparation of the Sample

**6.2.1** Extraction of Meat Samples

To the homogenised meat samples (labelled A - J), add a total of 90 ml PBS from a measuring cylinder, (100 mls final volume). To achieve dispersal of the meat, add about 5 ml of this volume first and disperse the sample with a spatula. Add a further 5 ml and stir. When about 20 ml has been added in this way the remaining volume can be added in total. Replace the caps and shake vigorously for about 10 seconds to ensure complete dispersal of the sample. Allow to stand at room temperature for 1.5 hours and agitate for a few seconds every 15 minutes (not critical). Loosen the screw caps and allow the extracts to stand undisturbed for 30 minutes, (to allow for sedimentation of the solids). Withdraw about 5 ml of the aqueous phase with disposable Pasteur pipettes and place in the stoppered sample containers supplied. Store at +4°C until required (3 days max).

**6.3** Preparation of Kit Materials

**6.3.1** Wash Solution Concentrate

Dilute the wash solution concentrate 1 to 20 with distilled water. The complete contents (50 ml) of the bottle can be diluted to 1 litre or smaller quantities can be diluted as required.

**6.3.2** Kit Reagents

The kit reagents (and test extracts) must be at room temperature before the immunoassay is commenced. Remove the vials from the kit box and leave on the bench for about 2 hours to equilibrate. Invert each vial several times before use to mix contents; **DO NOT SHAKE**. When the assay is completed the vials should be returned to the kit box and stored at +4°C.

**6.4** ELISA Procedure

**6.4.1** Cut open one end of the foil laminated microwell bag and remove the plate. Check that the desiccant bag shows blue. Remove four 2 × 8 stripwells and replace with the desiccant in the foil bag. **RE-SEAL IMMEDIATELY** with a heat sealer or with the sticky tape supplied. It is important that the remaining wells are kept away from moisture.

Number the two 2 × 8 stripwells remaining with a felt pen and place in the middle of the frame.

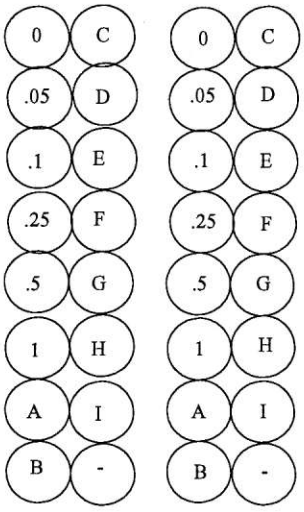
**6.4.2** Samples and standards must be treated in identical ways. With a 200 µl pipette, transfer 200 µl of each standard and each sample extract (in duplicate) into the appropriate wells, as indicated in the format given in Figure 1. Pipette in an orderly sequence using a fresh tip for each sample. The zero papain standard represents the sample blank.

This stage should be completed within 5 minutes.

Carefully cover with cling-film or a plate lid and allow to incubate at room temperature for 1 hour.

**FIGURE 1.**

**Format for Standard and Sample Extract in Microwells (Section 6.4.2)**




Standard Extracts:
0,0.05,0.1,0.25,0.5, 1 mg/kg

Test Extracts:
A-J

**6.4.3** Wash wells with diluted wash solution

The wash procedure is performed to remove unbound reagents from the wells. This involves filling the wells 3/4 full with wash solution and then emptying. This is repeated to give 6 washes in total. Washing can be performed most simply using one of the disposable Pasteur pipettes provided; emptying the wells by inversion over a sink and apply a flicking action to effectively remove well contents. Alternatively, an 8-channel pipette can be used to fill the wells or an automatic washing system can be used, if available.

After the 6th wash the wells should be patted onto a tissue to remove excess wash solution.

**6.4.4** With a 200 µl pipette, transfer 200 µl of CONJUGATE solution into all wells, (in the same order as before). Cover and allow to incubate at room temperature for 1 hour.

**6.4.5** Wash wells with diluted wash solution 6 times as before.

**6.4.6** With a 200 µl pipette, transfer 200 µl SUBSTRATE solution into all wells, (in the same order as before). To avoid contamination **DO NOT** pipette directly from the substrate vial. Transfer 8 ml into a clean disposable container and pipette from the latter. **DO NOT** return any unused substrate to the vial.

Allow the wells to incubate at room temperature for 10 to 12 minutes until the 1 mg/kg standard reaches 1.2 absorbance units, (relative to the zero papain standard). Chromophore development is temperature dependant; at a room temperature of 20°C this will take 12 min. **DO NOT** leave under the plate reader during the incubation stage as heat from the lamp will increase the reaction rate. Swirl the well holder fairly vigorously every 2 minutes to prevent product inhibition

occurring at the surface of the solid phase. This is achieved most easily with the frame on the bench.

**6.4.7** Pipette 50 µl of STOP solution into each well, (in the same order as before) and swirl the frame for 30 seconds to thoroughly mix the well contents.

NB There will be an increase of about 0.3 absorbance units for the top standard upon addition of stop solution because acidification enhances chromophore intensity.

Visually check that the zero papain wells show no colour.

**6.4.8** Immediately, measure the absorbance of each well on an ELISA plate reader fitted with a filter within the range 410 - 420 nm.

i.e. Zero the ELISA plate reader on air and measure the absorbance of each standard and test sample well.

## 7. Health and Safety

**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.**

**Particular care should be taken when handling the substrate solution as ABTS (2,2'-Azinobis(3-ethylbenzthiazoline sulphonic acid) is reported to be toxic.**

## 8 Expression of Results

Subtract the average of the standard zero papain readings from each other readings. Construct a calibration curve by plotting the averaged standard values against concentration of papain (mg/kg) on the log/linear graph paper provided. Use a 'Flexi-curve' to draw the line, which should pass through all 5 points on the graph.

As the standards and unknown meat samples have been extracted and assayed in identical ways the concentration of papain in the unknown samples is determined directly by interpolation on the standard curve and expressed as mg papain per kg meat.

## 9. General Notes

Kit components and test sample extracts should be kept at +4°C when not being used. However, they should be allowed to warm to room temperature before use. Allow at least 2 hours for temperature equilibration. Excess quantities of reagents are supplied with each kit. There are sufficient materials supplied to perform the pre-trial assay twice, (total of two 2 × 8 strips) and the trial assay twice, (total of four 2 × 8 strips). The immunoassay is technically simple to perform.

As with other immunoassay techniques the following general requirements apply:-

a. Take care not to cross-contaminate reagents.
b. Use a new pipette tip for each different sample, standard and reagent.
c. Do not allow pipette tips to come into contact with the surface of liquid within the wells.

**TABLE IX**
**Key to Tables III-VIII**

| | |
|---|---|
| a | Single result reported, values not used in calculation of mean, repeatability and reproducibility. |
| b | Result reported as ">", values not used in calculation of mean, repeatability and reproducibility. |
| r | Repeatability (within-laboratory variation). The value below which the absolute difference between two single test results obtained with the same method on identical test material under the **same conditions** may be expected to lie with 95% probability. |
| R | Reproducibility (between-laboratory variation). The value below which the absolute difference between two single test results obtained with the same method on identical test material under **different conditions** may be expected to lie with 95% probability. |
| $S_r$ | The standard deviation of the repeatability |
| $S_R$ | The standard deviation of the Reproducibility |
| $RSD_r$ | The relative standard deviation of the repeatability $S_r \times 100/x$ |
| $RSD_R$ | The relative standard deviation of the reproducibility $S_R \times 100/x$ |

APPENDIX II

| Standards | Absorbance | | Mean |
|---|---|---|---|
| mg/kg | 1 | 2 | |
| 0.05 | 0.14 | 0.14 | 0.14 |
| 0.10 | 0.27 | 0.26 | 0.27 |
| 0.25 | 0.61 | 0.58 | 0.60 |
| 0.50 | 0.99 | 0.95 | 0.97 |
| 1.00 | 1.39 | 1.36 | 1.38 |
| | | | |

| Sample | 1 | 2 | Mean | Papain mg/kg |
|---|---|---|---|---|
| $T_1$ | 0.86 | 0.84 | 0.85 | 0.41 |
| | | | | |
| | | | | |
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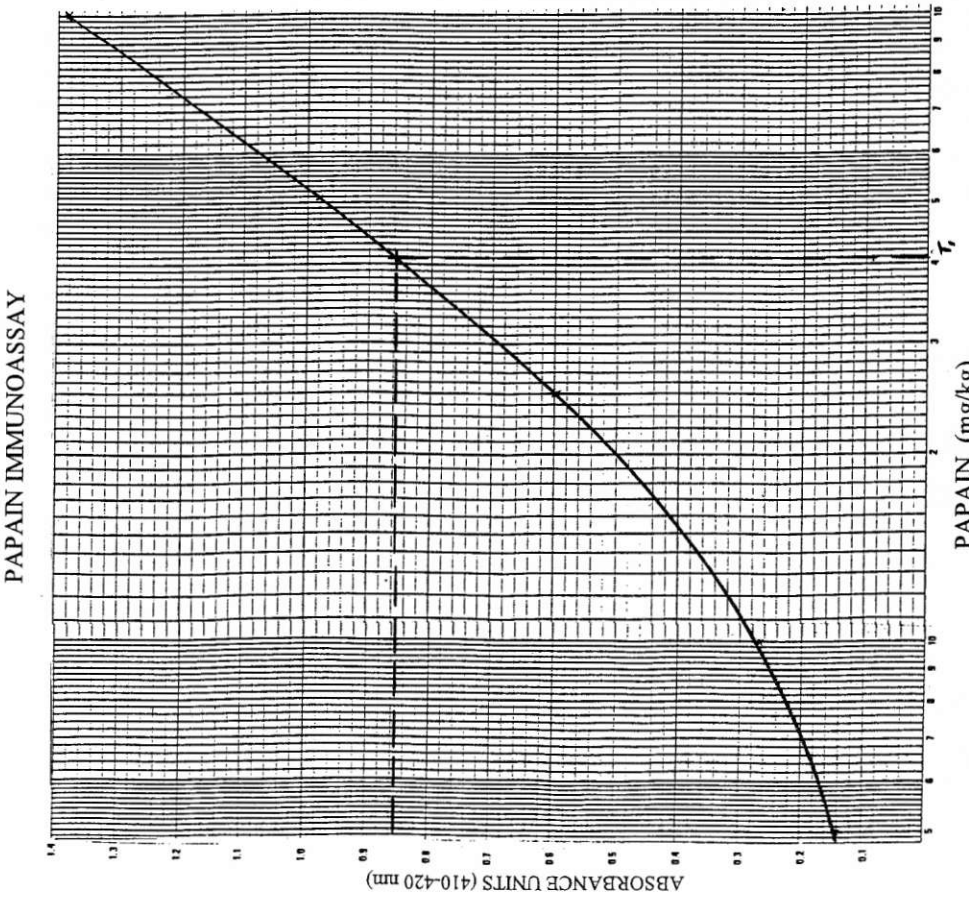

J. Assoc. Publ. Analysts, 28, 171-175

# MAFF VALIDATED METHODS FOR THE ANALYSIS OF FOODSTUFFS

**No. V 19**

## ACIDITY IN HONEY

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 allows the determination of the acidity of honey. It is the same as that described in CAC/12-1969, Codex Alimentarius Commission Recommended European Regional Standard for Honey.

### 2. Definition

**Acidity**: the content of acid (expressed in milli-equivalents of acid per kg) as determined by the method specified.

### 3. Principle

A plot of the neutralisation curve of honey is obtained by titration of a sample with sodium hydroxide. The acidity is calculated from the total titrant used at the equivalence point.

### 4. Reagents

All reagents should be of recognised analytical grade unless specified otherwise.

**4.1** Standard sodium hydroxide solution, 0.05 mol/l (carbonate-free).

**4.2** Water, carbon dioxide-free, prepared by boiling and cooling distilled water immediately prior to use.

### 5. Apparatus

**5.1** pH meter

**5.2** Magnetic stirrer

**5.3** Analytical balance

**5.4** Volumetric flask, 50 ml

**5.5** Beaker, 50 ml

**5.6** Pipettes, 25 ml

**5.7** Burette, capable of being read to 0.05 ml

### 6. Procedure

**6.1** Preparation of the sample for analysis

The mass of the sample presented to the laboratory for analysis shall be at least 200 g. The prepared sample shall always be kept in an air-tight and moisture-tight container.

**6.1.1** Liquid honey or pressed honey

 

If the sample is free from granulation, mix carefully by stirring or shaking. If the honey is granular, place in a closed container on a water bath, taking care not to immerse it, and heat for 30 min. at 60°C; further heat, if necessary, at 65°C until liquefication is complete, occasionally shaking the container. Mix carefully and allow to cool rapidly as soon as the sample liquefies. Do not heat honey which has to be used for the determination of hydroxymethylfurfural content or diastase activity. If the honey contains foreign matter (eg wax, twigs, bees or particles of honeycombs), heat the sample to 40°C on a waterbath and strain the honey through cheesecloth in a hot- water jacketed funnel before sample preparation.

**6.1.2** Comb honey

Remove the upper part of the combs, if they are sealed. Completely separate the honey from the combs by passing through a sieve; the mesh of the sieve is formed by wires woven to form square apertures of side 0.50 mm. If part of the wax or comb passes through the sieve, heat the sample as described under 6.1.1 and strain the honey through a filter. If the honey is granular, heat it until the wax liquefies, stir, allow to cool and remove the wax.

**6.2** Analysis of the prepared sample

**6.2.1** Accurately weigh approximately 5 g of honey. Dissolve in a few ml of water, transfer quantitatively into a 50 ml volumetric flask (**5.4**), and make up to volume with water. Pipette 25 ml from the flask into a beaker (**5.5**).

**6.2.2** Place a magnetic stirrer (**5.2**) in the beaker, stir the liquid gently and titrate potentiometrically with sodium hydroxide solution (**4.1**). Add the sodium hydroxide in increments of 0.05 ml only. Note the pH immediately after every addition of alkali solution.

**6.2.3** Plot the neutralisation curve of change of pH (on ordinate axis) against the volume of sodium hydroxide solution (on the abscissa). Determine from the graph the pH of neutralisation, ie at the inflection. Note that the volume of alkali solution plotted on the abscissa for a given change of pH should be the mean of the two volumes over which the pH change occurs.

## 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

The acidity, expressed as milli-equivalents of sodium hydroxide necessary to raise the pH of 1000 g of prepared honey to the neutralisation point, is given by:

$$\text{Acidity (meq/kg)} = \frac{1000 \times V \times M}{m}$$

where;

$m$ is the mass in g of the test sample, ie 0.5 × weight of sample taken (**6.1**);

$M$ is the molarity in mol/l of the sodium hydroxide solution;

$V$ is the volume in ml of the sodium hydroxide added to obtain the pH at the equivalence point.

## 9. References

**9.1** DW Lord, MJ Scotter, AD Whittaker and R Wood, J. Assoc. Publ. Analysts, 1989, **26**, 51-76.

**9.2** Ministry of Agriculture, Fisheries and Food, Food Safety Directorate, MAFF Validated Methods for the Analysis of Food, Introduction, General Considerations and Analytical Quality Control, J. Assoc. Publ. Analysts, 1992, **28**, 11-16.

## APPENDIX 1

### Analytical Quality Control

General principles of analytical quality control are outlined in protocol V.0 of the series[2].

### 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). At acidities above 12 meq/kg, r may be taken as 3 meq/kg. This corresponds to a relative standard deviation of repeatability (coefficient of variance of repeatability), $RSD_r$, of less than 9%. At lower acidities, the method appears less precise (r up to 5 meq/kg), with a $RSD_r$ of up to 25%.

### 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 (Table 1). Overall, R may be taken to be 6-9 meq/kg, corresponding to a relative standard deviation of reproducibility (coefficient of variance of reproducibility), $RSD_R$, of 20-50%. In particular, at higher acidities

(above 12 meq/kg), the better precision (R = 7 meq/kg, CV = 20%) can be expected.

## A3 Trueness (Bias)

Accuracy was not tested by spiking samples with known concentrations of acid. However, there is no reason to suspect systematic bias.

## 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 acidity of roughly 5 meq/kg for a single determination.

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

Participants in the collaborative trial each analysed eight samples of honey once (four samples from different countries in blind duplicate). The samples did not require preparation (**6.1**) before analysis.

Table 1 summarises the statistical data; the acidities are calculated from the titre and expressed as meq/kg.

**TABLE 1**
**Statistical Analysis of Acidity (meq/kg) in Honey Samples**

| Sample | 1/7 | 2/5 | 3/8 | 4/6 |
|---|---|---|---|---|
| Number of Laboratories retained after eliminating outliers | 18 | 19 | 17 | 17 |
| Number of Laboratories eliminated as outliers | 1 | 0 | 2 | 2 |
| Number of results accepted after eliminating outliers | 36 | 38 | 34 | 34 |
| LEVEL OF ANALYTE | | | | |
| Mean observed value $\bar{x}$ | 7.0 | 6.05 | 13.5 | 13.5 |
| REPEATABILITY | | | | |
| Standard Deviation $S_r$ | 1.68 | 1.04 | 0.71 | 0.36 |
| Relative Standard Deviation $RSD_r$(%) | 24 | 16 | 5.3 | 2.6 |
| Repeatability r [2.8 × $S_r$] | 4.7 | 2.9 | 2.0 | 2.6 |
| REPRODUCIBILITY | | | | |
| Standard Deviation $S_R$ | 3.0 | 2.2 | 2.5 | 2.5 |
| Relative Standard Deviation $RSD_R$(%) | 43 | 34 | 19 | 19 |
| Reproducibility R [2.8 × $S_R$] | 8.5 | 6.2 | 7.1 | 7.1 |

## 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, 28, 177-181

# MAFF VALIDATED METHODS FOR THE ANALYSIS OF FOODSTUFFS

**No. V 20**

## ASH IN HONEY

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 allows the determination of the ash content of honey; this is taken as a measure of total mineral content. It is the same in principle as that described in CAC/12-1969, Codex Alimentarius Commission Recommended European Regional Standard for Honey.

### 2. Definition

**Ash content**: the content of ash as determined by the method specified.

### 3. Principle

The residual mass of a test portion is determined gravimetrically after incineration in an oxidising atmosphere at 600°C and calculated as a percentage by mass of the sample.

### 4. Reagents

All reagents should be of recognised analytical grade unless specified otherwise.

**4.1** Olive oil, food grade.

**4.2** Dilute hydrochloric acid, approximately 7 g per 100 ml. Carefully add, with stirring, 100 ml of hydrochloric acid (**4.2.1**) to 500 ml of water and mix.

**4.2.1** Hydrochloric acid, concentrated, (HCl, density 1.64 g/ml)

### 5. Apparatus

**5.1** Incineration dishes, made of platinum or silica.

**5.2** Electric muffle furnace, air-ventilated, temperature controlled by thermostat at 600°C with a differential no larger than 25°C, fitted with a pyrometer.

**5.3** Infra-red lamp

**5.4** Desiccator, containing an efficient desiccant, e.g. dried silica gel.

### 6. Procedure

**6.1** Preparation of the sample for analysis

The mass of the sample presented to the laboratory for analysis shall be at least 200 g. The prepared sample shall always be kept in an air-tight and moisture-tight container.

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**6.1.1** Liquid honey or pressed honey

If the sample is free from granulation, mix carefully by stirring or shaking. If the honey is granular, place in a closed container on a water bath, taking care not to immerse it, and heat for 30 min. at 60°C. If necessary, further heat at 65°C until liquefication is complete, occasionally shaking the container. Mix carefully and allow to cool rapidly as soon as the sample liquefies. Do not heat honey which has to be used for the determination of hydroxymethylfurfural content or diastase activity. If the honey contains foreign matter (eg wax, twigs, bees or particles of honeycombs), heat the sample to 40°C on a waterbath and strain the honey through cheesecloth in a hot- water jacketed funnel before sample preparation.

**6.1.2** Comb honey

Remove the upper part of the combs, if they are sealed. Completely separate the honey from the combs by passing through a sieve; the mesh of the sieve is formed by wires woven to form square apertures of side 0.50 mm. If part of the wax or comb passes through the sieve, heat the sample as described under 6.1.1 and strain the honey through a filter. If the honey is granular, heat it until the wax liquefies, stir, allow to cool and remove the wax.

**6.2** Preparation of the incineration dish

Clean the incineration dish (**5.1**), whether new or not, with boiling dilute hydrochloric acid (**4.2**). Rinse it free from acid with large quantities of water. Heat it for 30 min. in the muffle furnace (**5.2**). Remove it from the furnace, allow it to cool to ambient temperature in the desiccator (**5.4**) and weigh it to the nearest 0.1 mg ($m_1$).

**6.3** Analysis of the prepared sample

**6.3.1** Weigh into the prepared incineration dish (**6.2**), to the nearest 1 mg, about 5-10 g of honey ($m_0$).

**6.3.2** Place the dish and contents (**6.3.1**) in the muffle furnace (**5.2**) and heat gently until the sample becomes black and dry. Care must be taken to avoid risk of loss through foaming and excessive swelling of the mass. An infra- red lamp (**5.3**) may be used to aid carbonisation of the sample prior to putting it in the muffle furnace; such initial charring may be essential to prevent excessive foaming. The addition of a few drops of olive oil (**4.1**) may also help to prevent excessive swelling.

**6.3.3** Ignite the dish at 600°C until no further apparent change in colour of the residue ash occurs.

**6.3.4** Remove the dish from the furnace, place it in the desiccator (**5.4**) and allow it to cool to ambient temperature.

**6.3.5** Weigh the dish and residue to the nearest 0.1 mg.

**6.3.6** Repeat operations 6.3.3, 6.3.4 and 6.3.5 until the difference between two successive weighings is less than 0.1 mg. Designate the final weight $m_2$.

## 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

The ash content, calculated as a percentage by mass of the prepared sample, is given by:

$$\% \text{ Ash content} = 100 \times (m_2 - m_1) / m_0$$

where;

$m_0$ is the mass of the test portion, in g (**6.3.1**);

$m_1$ is the mass of the prepared incineration dish, in g (**6.2**);

$m_2$ is the mass of the incineration dish and the residue, in g (**6.3.6**).

## 9. References

**9.1** DW Lord, MJ Scotter, AD Whittaker and R Wood, J. Assoc. Publ. Analysts, 1989, **26**, 51-76.

**9.2** Ministry of Agriculture, Fisheries and Food, Food Safety Directorate, MAFF Validated Methods for the Analysis of Food, Introduction, General Considerations and Analytical Quality Control, J. Assoc. Publ. Analysts, 1992, **28**, 11-16.

## APPENDIX 1

### Analytical Quality Control

General principles of analytical quality control are outlined in protocol V.0 of the series[2].

## 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). Within the range 0.05-0.2% ash, r may be taken as 0.05% ash. This corresponds to a relative standard deviation of repeatability (coefficient of variance of repeatability), $RSD_r$, of 7-29%. The value of r may be somewhat higher (0.1% ash) at higher ash levels (above 0.2% ash).

## 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 (Table 1). Overall, R may be taken to be 0.05-0.14% ash, corresponding to a relative standard deviation of reproducibility (coefficient of variance of reproducibility), $RSD_R$ of 22-40%.

## A3 Trueness (Bias)

Accuracy was not tested by spiking samples with known concentrations of minerals. However, there is no reason to suspect systematic bias.

## 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 ash content of roughly 0.04% ash for a single determination.

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

Participants in the collaborative trial each analysed eight samples of honey once (four samples from different countries in blind duplicate). The samples did not require preparation (**6.1**) before analysis.

Table 1 summarises the statistical data; the levels of ash are expressed as a percentage by mass of the sample.

**TABLE 1**
**Statistical Analysis of the % Ash in Honey Samples**

| Sample | 1/7 | 2/5 | 3/8 | 4/6 |
|---|---|---|---|---|
| Number of Laboratories retained after eliminating outliers | 19 | 17 | 19 | 19 |
| Number of Laboratories eliminated as outliers | 0 | 2 | 0 | 0 |
| Number of results accepted after eliminating outliers | 38 | 34 | 38 | 38 |
| LEVEL OF ANALYTE | | | | |
| Mean observed value $\bar{x}$ | 0.23 | 0.05 | 0.20 | 0.08 |
| REPEATABILITY | | | | |
| Standard Deviation $S_r$ | 0.03 | 0.01 | 0.01 | 0.02 |
| Relative Standard Deviation $RSD_r$(%) | 14 | 29 | 7 | 22 |
| Repeatability r [2.8 × $S_r$] | 0.09 | 0.04 | 0.04 | 0.05 |
| REPRODUCIBILITY | | | | |
| Standard Deviation $S_R$ | 0.05 | 0.02 | 0.05 | 0.03 |
| Relative Standard Deviation $RSD_R$(%) | 22 | 36 | 23 | 40 |
| Reproducibility R [2.8 × $S_R$] | 0.14 | 0.05 | 0.13 | 0.09 |

## 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, 28, 183-187

# MAFF VALIDATED METHODS FOR THE ANALYSIS OF FOODSTUFFS

No. V 21

## MOISTURE IN HONEY

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 allows the determination of the moisture content of clear and normally coloured honeys. It is the same in principle as that described in CAC/12-1969, Codex Alimentarius Commission Recommended European Regional Standard for Honey.

### 2. Definition

**Moisture content**: the content of moisture as determined by the method specified.

### 3. Principle

The refractive index of a test portion is determined at 20°C and converted into moisture content by reference to tables showing concentration as a function of refractive index.

### 4. Reagents

None.

### 5. Apparatus

**5.1** Refractometer, capable of being read to unity in the fourth decimal place over the refractive index range 1.4700 to 1.5100, provided with means for the circulation of water about the prisms and a thermometer, the bulb of which is immersed in the circulating water stream. The thermometer shall have a certificate of accuracy at 20°C.

**5.2** Light source, for the refractometer (**5.1**) consisting of a sodium lamp of the type recommended by, and adjusted in accordance with the instructions of, the manufacturer of the refractometer.

**5.3** Water bath, controlled by a thermostat at 20°C with a differential no larger than 0.5°C, fitted with a pump for circulating water about the prisms of the refractometer (**4.1**).

**5.4** Glass or plastic rod, with an angled, flattened end, as required for applying the test portion to the prism of the refractometer (**4.1**).

### 6. Procedure

**6.1** Preparation of the sample for analysis

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The mass of the sample presented to the laboratory for analysis shall be at least 200 g. The prepared sample shall always be kept in an air- tight and moisture- tight container.

**6.1.1** Liquid honey or pressed honey

If the sample is free from granulation, mix carefully by stirring or shaking. If the honey is granular, place in a closed container on a water bath, taking care not to immerse it, and heat for 30 min. at 60°C. If necessary, further heat at 65°C until liquefication is complete, occasionally shaking the container. Mix carefully and allow to cool rapidly as soon as the sample liquefies. Do not heat honey which has to be used for the determination of hydroxymethylfurfural content or diastase activity. If the honey contains foreign matter (eg wax, twigs, bees or particles of honeycombs), heat the sample to 40°C on a waterbath and strain the honey through cheesecloth in a hot- water jacketed funnel before sample preparation.

**6.1.2** Comb honey

Remove the upper part of the combs, if they are sealed. Completely separate the honey from the combs by passing through a sieve; the mesh of the sieve is formed by wires woven to form square apertures of side 0.50 mm. If part of the wax or comb passes through the sieve, heat the sample as described under 6.1.1 and strain the honey through a filter. If the honey is granular, heat it until the wax liquefies, stir, allow to cool and remove the wax.

**6.2** Analysis of the prepared sample

Measure the refractive index of the prepared sample at 20°C in the refractometer (**5.1**).

## 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

Calculate the moisture content using the refractive index values shown in the Conversion Table below. The moisture is expressed as a percentage by mass of the prepared sample.

**8.2** Corrections

The following correction to the refractometer reading must be used if a temperature other than 20°C is employed.

**8.2.1** Temperature above 20°C: add 0.00023 per °C.

**8.2.2** Temperature below 20°C: subtract 0.00023 per °C.

## 9. References

**9.1** DW Lord, MJ Scotter, AD Whittaker and R Wood, J. Assoc. Publ. Analysts, 1989, **26**, 51- 76.

**9.2** Ministry of Agriculture, Fisheries and Food, Food Safety Directorate, MAFF Validated Methods for the Analysis of Food, Introduction, General Considerations and Analytical Quality Control, J. Assoc. Publ. Analysts, 1992, **28**, 11-16.

## APPENDIX 1

## Conversion Table for Estimation of Moisture Content

| Refractive index (20°C) | Moisture content % | Refractive index (20°C) | Moisture content % |
|---|---|---|---|
| 1.5044 | 13.0 | 1.4885 | 19.2 |
| 1.5038 | 13.2 | 1.4880 | 19.4 |
| 1.5033 | 13.4 | 1.4875 | 19.6 |
| 1.5028 | 13.6 | 1.4870 | 19.8 |
| 1.5023 | 13.8 | 1.4865 | 20.0 |
| 1.5018 | 14.0 | 1.4860 | 20.2 |
| 1.5012 | 14.2 | 1.4855 | 20.4 |
| 1.5007 | 14.4 | 1.4850 | 20.6 |
| 1.5002 | 14.6 | 1.4845 | 20.8 |
| 1.4997 | 14.8 | 1.4840 | 21.0 |
| 1.4992 | 15.0 | 1.4835 | 21.2 |
| 1.4987 | 15.2 | 1.4830 | 21.4 |
| 1.4982 | 15.4 | 1.4825 | 21.6 |
| 1.4976 | 15.6 | 1.4820 | 21.8 |
| 1.4971 | 15.8 | 1.4815 | 22.0 |
| 1.4969 | 16.0 | 1.4810 | 22.2 |
| 1.4961 | 16.2 | 1.4805 | 22.4 |
| 1.4956 | 16.4 | 1.4800 | 22.6 |
| 1.4951 | 16.6 | 1.4795 | 22.8 |
| 1.4946 | 16.8 | 1.4790 | 23.0 |
| 1.4940 | 17.0 | 1.4785 | 23.2 |
| 1.4935 | 17.2 | 1.4780 | 23.4 |
| 1.4930 | 17.4 | 1.4775 | 23.6 |
| 1.4925 | 17.6 | 1.4770 | 23.8 |
| 1.4920 | 17.8 | 1.4765 | 24.0 |
| 1.4915 | 18.0 | 1.4760 | 24.2 |
| 1.4910 | 18.2 | 1.4755 | 24.4 |
| 1.4905 | 18.4 | 1.4750 | 24.6 |
| 1.4900 | 18.6 | 1.4745 | 24.8 |
| 1.4895 | 18.8 | 1.4740 | 25.0 |
| 1.4890 | 19.0 | | |

## APPENDIX 2

## Analytical Quality Control

General principles of analytical quality control are outlined in protocol V.0 of the series[2].

### 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). At moisture levels of about 17%, r may be taken to be 0.5% moisture. This corresponds to a relative standard deviation of repeatability (coefficient of variance of repeatability), $RSD_r$, of 1%.

### 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 (Table 1). Overall, R may be taken to be 0.9% moisture, corresponding to a relative standard deviation of reproducibility (coefficient of variance of reproducibility), $RSD_R$, of 2%.

### A3 Trueness (Bias)

Accuracy was not tested by spiking samples with known concentrations of water. However, there is no reason to suspect systematic bias.

### 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 0.3% moisture for a single determination.

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

Participants in the collaborative trial each analysed eight samples of honey once (four samples from different countries in blind duplicate). The samples did not require preparation (**6.1**) before analysis.

Table 1 summarises the statistical data; the levels of moisture are expressed as a percentage by mass of the sample.

**TABLE 1**

**Statistical Analysis of the % Moisture in Honey**

| Sample | 1/7 | 2/5 | 3/8 | 4/6 |
|---|---|---|---|---|
| Number of Laboratories retained after eliminating outliers | 18 | 18 | 16 | 15 |
| Number of Laboratories eliminated as outliers | 1 | 1 | 3 | 5 |
| Number of results accepted after eliminating outliers | 36 | 36 | 32 | 30 |
| LEVEL OF ANALYTE | | | | |
| Mean observed value $\bar{x}$ | 15.9 | 17.8 | 17.3 | 16.0 |
| REPEATABILITY | | | | |
| Standard Deviation $S_r$ | 0.1 | 0.19 | 0.08 | 0.06 |
| Relative Standard Deviation $RSD_r$(%) | 0.6 | 1.1 | 0.4 | 0.4 |
| Repeatability r [2.8 × $S_r$] | 0.28 | 0.53 | 0.21 | 0.17 |
| REPRODUCIBILITY | | | | |
| Standard Deviation $S_R$ | 0.33 | 0.31 | 0.14 | 0.20 |
| Relative Standard Deviation $RSD_R$(%) | 2.1 | 1.7 | 0.8 | 1.2 |
| Reproducibility R [2.8 × $S_R$] | 0.92 | 0.87 | 0.40 | 0.55 |

## 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, 28, 189-193

# MAFF VALIDATED METHODS FOR THE ANALYSIS OF FOODSTUFFS

## No. V 22

## WATER-INSOLUBLE SOLIDS IN HONEY

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 allows the determination of the water-insoluble solids content of honey. It is the same in principle as that described in CAC/12-1969, Codex Alimentarius Commission Recommended European Regional Standard for Honey.

### 2. Definition

**Water- insoluble solids content**: the content of water- insoluble solids as determined by the method specified.

### 3. Principle

The water- insoluble solids content is determined gravimetrically, after filtration, of the honey in solution, and drying the residue for 1 hr. at 135°C.

### 4. Reagents

All reagents should be of recognised analytical grade unless specified otherwise.

**4.1** Phloroglucinol solution, 1% *m/V* in ethanol

**4.2** Sulphuric acid, concentrated

### 5. Apparatus

**5.1** Analytical balance, capable of weighing to 0.1 mg.

**5.2** Sintered glass crucible, pore size 15-40 μm.

**5.3** Drying oven, electrically heated, thermostatically controlled at a temperature of 135 ± 1°C.

**5.4** Desiccator, containing an efficient desiccant, e.g. dried silica gel.

### 6. Procedure

**6.1** Preparation of the sample for analysis

The mass of the sample presented to the laboratory for analysis shall be at least 200 g. The prepared sample shall always be kept in an air-tight and moisture-tight container.

**6.1.1** Liquid honey or pressed honey

If the sample is free from granulation, mix carefully by stirring or shaking. If the honey is granular, place in a closed container on a

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water bath, taking care not to immerse it, and heat for 30 min. at 60°C. If necessary, further heat at 65°C until liquefication is complete, occasionally shaking the container. Mix carefully and allow to cool rapidly as soon as the sample liquefies. Do not heat honey which has to be used for the determination of hydroxymethylfurfural content or diastase activity. If the honey contains foreign matter (eg wax, twigs, bees or particles of honeycombs), heat the sample to 40°C on a waterbath and strain the honey through cheesecloth in a hot- water jacketed funnel before sample preparation.

**6.1.2** Comb honey

Remove the upper part of the combs, if they are sealed. Completely separate the honey from the combs by passing through a sieve; the mesh of the sieve is formed by wires woven to form square apertures of side 0.50 mm. If part of the wax or comb passes through the sieve, heat the sample as described under 6.1.1 and strain the honey through a filter. If the honey is granular, heat it until the wax liquefies, stir, allow to cool and remove the wax.

**6.2** Analysis of the prepared sample

**6.2.1** Dry a sintered glass crucible (**5.2**) for 1 hr. at 135°C in the oven (**5.3**), allow to cool in the desiccator (**5.4**) and weigh to an accuracy of 0.1 mg ($m_1$)

**6.2.2** Accurately weigh about 20 g of honey ($m_0$) and dissolve it in a suitable volume (ca 200 ml) of water at 80°C; mix well.

**6.2.3** Filter through the previously dried and weighed sintered glass crucible (**6.2.1**).

**6.2.4** Wash through the crucible carefully with water at 80°C until free from sugars. Thorough washing with warm water is essential; use Mohr's test (**6.2.5**) to check that washing is complete.

**6.2.5** Mohr's test

Add phloroglucinol solution (**4.1**) to the filtrate in a test tube. Mix. Run a few drops of concentrated sulphuric acid (**4.2**) down the side of the tube; colour is produced at the interface if sugars are present.

**6.2.6** Dry the crucible for 1 hr. at 135°C in the oven (**5.3**), allow to cool in the desiccator (**5.4**) and weigh to an accuracy of 0.1 mg.

**6.2.7** Repeat the drying until constant weight ($m_2$) is obtained.

## 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

The water- insoluble solids content, calculated as a percentage by mass of the prepared sample, is given by:

$$\% \text{ water- insoluble solids content} = 100 \times (m_2 - m_1)/m_0$$

where:

$m_0$ is the mass of the test sample, in g (**6.2.2**);

$m_1$ is the mass of the dried sintered glass crucible in g (**6.2.1**);

$m_2$ is the mass of the dried crucible and filtered solids, in g (**6.2.7**).

## 9. References

**9.1** DW Lord, MJ Scotter, AD Whittaker and R Wood, J. Assoc. Publ. Analysts, 1989, **26**, 51- 76.

**9.2** Ministry of Agriculture, Fisheries and Food, Food Safety Directorate, MAFF Validated Methods for the Analysis of Food, Introduction, General Considerations and Analytical Quality Control, J. Assoc. Publ. Analysts, 1992, **28**, 11-16.

## APPENDIX 1

## Analytical Quality Control

General principles of analytical quality control are outlined in protocol V.0 of the series[(2)].

### 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). Within the range 0.01- 0.03% water-insoluble solids, r may be taken as 0.02% solids. This corresponds to a relative standard deviation of repeatability (coefficient of variance of repeatability), $RSD_r$ of 24- 71%. Similar or better precision may be expected at levels up to 0.5% solids and beyond.

### 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 (Table 1). Overall, R may be taken to be 0.026% water-insoluble solids, corresponding to a relative standard deviation of reproducibility (coefficient of variance of reproducibility), $RSD_R$, of 31-93%.

### A3 Trueness (Bias)

Accuracy was not tested by spiking samples with known concentrations of water-insoluble solids. However, there is no reason to suspect systematic bias.

## 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 water-insoluble solids content of roughly 0.02% solids for a single determination.

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

Participants in the collaborative trial each analysed eight samples of honey once (four samples from different countries in blind duplicate). The samples did not require preparation (**6.1**) before analysis.

Table 1 summarises the statistical data; the levels of water-insoluble solids are expressed as a percentage by mass of the sample.

**TABLE 1**

**Statistical Analysis of the % Water-insoluble Solids (*m/m* %) in Honey Samples**

| Sample | 1/7 | 2/5 | 3/8 | 4/6 |
|---|---|---|---|---|
| Number of Laboratories retained after eliminating outliers | 15 | 16 | 17 | 16 |
| Number of Laboratories eliminated as outliers | 4 | 3 | 2 | 3 |
| Number of results accepted after eliminating outliers | 30 | 32 | 34 | 32 |
| LEVEL OF ANALYTE | | | | |
| Mean observed value $\bar{x}$ | 0.02 | 0.01 | 0.03 | 0.01 |
| REPEATABILITY | | | | |
| Standard Deviation $S_r$ | 0.01 | 0.01 | 0.01 | 0 |
| Relative Standard Deviation $RSD_r$(%) | 27 | 63 | 26 | 32 |
| Repeatability r [2.8 xh $S_r$] | 0.02 | 0.02 | 0.02 | 0.01 |
| REPRODUCIBILITY | | | | |
| Standard Deviation $S_R$ | 0.01 | 0.01 | 0.01 | 0.01 |
| Relative Standard Deviation $RSD_R$(%) | 36 | 63 | 26 | 84 |
| Reproducibility R [2.8 xh $S_R$] | 0.02 | 0.02 | 0.02 | 0.03 |

## 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, 28, 195-199

# MAFF VALIDATED METHODS FOR THE ANALYSIS OF FOODSTUFFS

**No. V23**

## HYDROXYMETHYLFURFURAL (HMF) IN HONEY

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 allows the determination of the HMF content of honey. It is the same in principle as that described in CAC/12-1969, Codex Alimentarius Commission Recommended European Regional Standard for Honey, and is based on ref. 9.2.

### 2. Definition

**The hydroxymethylfurfural content**: the HMF content as determined by the method specified.

### 3. Principle

Spectrophotometric determination using barbituric acid and *p*-toluidine solutions.

### 4. Reagents

All reagents should be of recognised analytical grade unless specified otherwise.

**4.1** Barbituric acid solution

**4.1.1** Transfer 500 mg of barbituric acid to a 100 ml volumetric flask using 70 ml of water. Place the flask on a very hot water bath until the barbituric acid dissolves, allow to cool and make up to volume with water.

**4.1.2** Barbituric acid

**4.2** *para*-Toluidine solution

**4.2.1** Weigh 10.0 g of *p*-toluidine and dissolve in approximately 50 ml of iso-propanol by heating gently on a water bath. Transfer the solution to a 100 ml volumetric flask with *iso*-propanol and add 10 ml of glacial acetic acid. Allow to cool and make up to the calibration mark with *iso*-propanol. Do not use for at least 24 hr. Store the solution in the dark. Care should be taken when handling *p*-toluidine.

**4.2.2** *iso*-Propanol

**4.2.3** *p*-Toluidine

**4.2.4** Acetic acid, glacial

**4.3** Water, oxygen-free.

Bubble oxygen-free nitrogen into boiling water for several minutes. Allow the water to cool before use.

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**4.4** Hydroxymethylfurfural, pure, for preparation of standard solution (**6.2**).

## 5. Apparatus

**5.1** Spectrophotometer, calibrated to read at 550 nm.

**5.2** Volumetric flasks, 50 ml and 100 ml capacity.

**5.3** Pipettes, 1 ml, 2 ml and 5 ml.

## 6. Procedure

**6.1** Preparation of the sample for analysis

The mass of the sample presented to the laboratory for analysis shall be at least 200 g. The prepared sample shall always be kept in an air-tight and moisture-tight container.

**6.1.1** Liquid honey or pressed honey

Mix carefully by stirring or shaking without heating.

**6.1.2** Comb honey

Remove the upper part of the combs, if they are sealed. Completely separate the honey from the combs without heating, if practicable by passing through a sieve; the mesh of the sieve is formed by wires woven to form square apertures of side 0.50 mm.

**6.2** Preparation of the sample solution

Weigh 10 g sample of prepared honey (**6.1**) and dissolve it without heating in 20 ml of oxygen-free water (**4.3**). Wash the entire contents into a 50 ml volumetric flask (**5.2**) and make up to volume with water (**4.3**); designate "honey solution". The solution should be analysed as soon as it has been prepared.

**6.3** Photometric determination

**6.3.1** Sample determination

Take two test tubes and pipette into each of them 2.0 ml of honey solution (**6.2**); then add to each tube 5.0 ml of the *p*-toluidine solution (**4.2**). Pipette into one of the tubes (blank) 1.0 ml of water (**4.3**) and into the other (sample) 1.0 ml of barbituric acid solution (**4.1**). Agitate both tubes. Add the reagents quickly so as to complete the operation within a minute or two. Read off the extinction of the solution in the sample tube compared with the blank tube at 550 nm using a 1 cm cell, as soon as the maximum absorbance value is reached.

**6.3.2** Standard determination

The method should be calibrated using a standard solution of HMF (**4.4**). Check the purity of the HMF standard by spectrophotometric assay at 284 nm, where the value of E (molar) is 16.830. Prepare a solution of HMF in water so that a 10 µg standard of HMF in 2 ml of water can be used in place of the honey solution in 6.3.1. Carry out the colour reaction as in 6.3.1 and measure the absorbance as in 6.3.1.

## 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

The level of HMF is calculated and expressed as mg of HMF per kg of prepared honey.

**8.1** If the method is not standardised, an approximate value for HMF level (mg HMF per kg honey) is given by:

$$\text{HMF content (mg/kg)} = \frac{\text{Absorbance} \times 192}{\text{Cell path length (cm)}}$$

**8.2** Using a standard determination, the HMF level (mg of HMF per kg of honey) is given by:

$$\text{HMF content (mg/kg)} = \frac{\text{Absorbance of sample solution} \times 25}{\text{Absorbance of standard solution}}$$

## 9. References

**9.1** DW Lord, MJ Scotter, AD Whittaker and R Wood, J. Assoc. Publ. Analysts, 1989, **26**, 51-76.

**9.2** JH Turner, PA Roberts, CL Barrich and RH Cotton, Anal. Chem., 1954, **26**, 898.

**9.3** Ministry of Agriculture, Fisheries and Food, Food Safety Directorate, MAFF Validated Methods for the Analysis of Food, Introduction, General Considerations and Analytical Quality Control, J. Assoc. Publ. Analysts, 1992, **28**, 11-16.

### APPENDIX 1

### Analytical Quality Control

General principles of analytical quality control are outlined in protocol V.0 of the series[3].

A number of laboratories taking part in the collaborative trial of this method experienced some difficulty in obtaining the intralaboratory precision expected. Moreover, the substantial observed differences between repeatability and reproducibility values suggest that interlaboratory precision is not altogether satisfactory. Further investigation is required, and the possibility of an alternative method must be considered; meanwhile, the method may be used within the constraints of the observed precision (Table 1), but cannot be recommended for enforcement purposes.

## 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). Overall, r may be taken as 9 mg/kg, though better precision might be expected. When analysing honey with HMF content above 20 mg/kg, this corresponds to a relative standard deviation of repeatability (coefficient of variance of repeatability), $RSD_r$, of less than 7%. However, at lower HMF contents the method becomes less precise ($RSD_r$ up to 30%).

## 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 (Table 1). Overall, R may be taken as 30 mg/kg, corresponding to a relative standard deviation of reproducibility (coefficient of variance of reproducibility), $RSD_R$, of up to 34%.

## A3 Trueness (Bias)

Accuracy was not tested by spiking samples with known concentrations of HMF. However, there is no reason to suspect systematic bias.

## 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 acidity of roughly 9 mg of HMF per kg of honey for a single determination.

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

Participants in the collaborative trial each analysed eight samples of honey once (four samples from different countries in blind duplicate). The samples did not require preparation (**6.1**) before analysis.

Table 1 summarises the statistical data; the HMF levels are expressed as mg of HMF per kg of honey.

**TABLE 1**

**Statistical Analysis of Hydroxymethylfurfural (HMF) in Honey Samples**

| Sample | 1/7 | 2/5 | 3/8 | 4/6 |
|---|---|---|---|---|
| Number of Laboratories retained after eliminating outliers | 18 | 14 | 17 | 17 |
| Number of Laboratories eliminated as outliers | 1 | 5 | 2 | 2 |
| Number of results accepted after eliminating outliers | 36 | 28 | 34 | 34 |
| LEVEL OF ANALYTE | | | | |
| Mean observed value $\bar{x}$ | 11.4 | 21.2 | 41.8 | 54.8 |
| REPEATABILITY | | | | |
| Standard Deviation $S_r$ | 3.4 | 0.89 | 2.9 | 1.6 |
| Relative Standard Deviation $RSD_r$(%) | 29 | 4 | 7 | 3 |
| Repeatability r [2.8 xh $S_r$] | 9.4 | 2.5 | 8.2 | 4.6 |
| REPRODUCIBILITY | | | | |
| Standard Deviation $S_R$ | 3.9 | 2.5 | 10.0 | 7.2 |
| Relative Standard Deviation $RSD_R$(%) | 34 | 12 | 24 | 13 |
| Reproducibility R [2.8 xh $S_R$] | 11.0 | 7.0 | 27.9 | 20.2 |

**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, 28, 201-205

# MAFF VALIDATED METHODS FOR THE ANALYSIS OF FOODSTUFFS

**No. V 24**

## INSOLUBLE MATTER IN INSTANT COFFEE

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 allows the determination of the water-insoluble matter content of instant coffee.

The precision of the method is acceptable at levels above 50 mg of insoluble matter per 100 g of sample (i.e. 0.05% *m/m*); however, the "natural" levels of insoluble matter in instant coffees should be considerably less than this concentration. The method will therefore have limited application as a statutory procedure, though it will discriminate against gross adulteration of samples by insoluble material.

### 2. Definition

**Insoluble matter content:** the content of the water-insoluble matter as determined by the method specified.

### 3. Principle

Test portions of the sample are dissolved in water and filtered through a specified filtering disc with square apertures of 100 μm × 100 μm; the residue trapped by the disc is dried and determined gravimetrically.

### 4. Reagents

None.

### 5. Apparatus

**5.1** Filtration apparatus, illustrated in Fig. 1 and consisting of the following parts. Apparatus 5.1.2 to 5.1.5 is not specified exactly, but it is essential that a sieve conforming to the specifications given in 5.1.1 is always employed.

**5.1.1** Metallic filtering disc, micro-precision sieve, made from nickel by electroforming, with support screen: diameter 29 mm, massive periphery ca. 3 mm wide, 70 μm thick, square holes of side 100 μm, 159 mesh.

**5.1.2** Borosilicate glass cup

**5.1.3** Borosilicate glass tulip

**5.1.4** Flange for heading joint

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**5.1.5** Sealing rings

**5.2** Tweezers, for microscope slides.

**5.3** Precision balance, reading to 0.01 mg.

**5.4** Filtering installation, for reduced pressure filtration.

**5.5** Oven, without forced ventilation, thermostatically controlled at 103 ± 2°C.

**5.6** Desiccator, containing freshly activated silica gel (or an equivalent desiccant) with a moisture content indicator.

**5.7** Analytical balance

## 6. Procedure

**6.1** Preparation of the filtration apparatus

Spread glass beads (diameter about 4 mm) all over the bottom of a small petri dish (diameter about 60 mm). Place the clean filter disc into the petri dish and dry for 30 min. in an oven at 103 ± 2°C. Cool to room temperature in a desiccator and weigh the disc to the nearest 0.1 mg ($M_0$).

Set up the filtration apparatus according to Fig. 1, and fix it onto the filtering flask by means of a rubber joint.

**6.2** Preparation of the solution

Accurately weigh, to the nearest 1 mg, about 5 g ($M_1$) of sample into an 800 ml beaker. Add 500 ml of boiling water, and stir for 10- 15 sec. with a glass rod with rounded ends.

**6.3** Filtration

Pour the solution as hot as possible into the filtration apparatus. Filter at room pressure; use of vacuum does not aid filtration. Pat the top of the cup with the palm of the hand to facilitate the filtration.

Rinse the beaker and the cup inside with about 200 ml of hot water, so that all insoluble particles are collected on the filtering disc. Finally, apply vacuum to remove the water held back on the disc.

Carefully dismantle the filtration apparatus in order to take out the filtering disc without losing insoluble matter. Place the filtering disc into the petri dish and dry it for 30 min. in an oven at 103±2°C. Allow it to cool to room temperature in a desiccator and weigh the disc to the nearest 0.1 mg ($M_2$).

## 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

The content of insoluble matter, calculated as a percentage by mass of the prepared sample, is given by:

$$\%\ (m/m)\ \text{Insoluble matter} = 100 \times (M_2 - M_0) / M_1$$

where:

$M_0$ is the mass of the clean, dry sieve disc, in g (**6.1**);

$M_1$ is the mass of the test portion, in g (**6.2**);

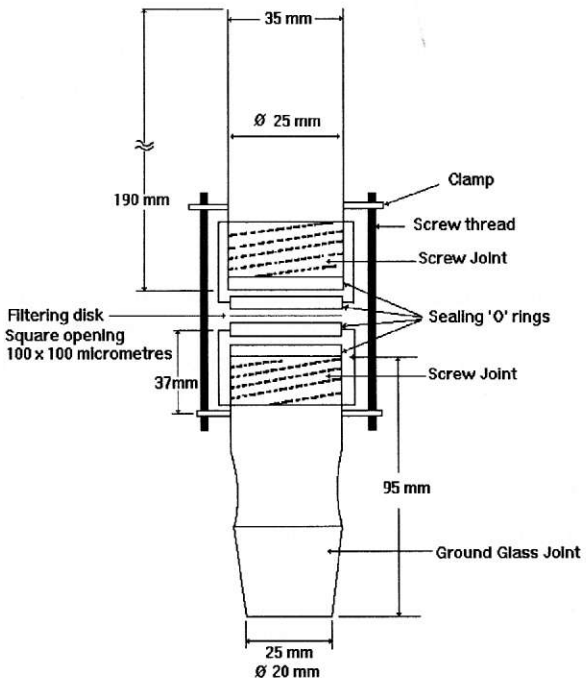
$M_2$ is the mass of the sieve disc and insoluble matter, in g (**6.3**).

## 9. References

**9.1** SL Reynolds, SA Thorpe and R Wood, J. Assoc. Publ. Analysts, 1983, 21, 47- 52.

**9.2** Ministry of Agriculture, Fisheries and Food, Food Safety Directorate, MAFF Validated Methods for the Analysis of Food, Introduction, General Considerations and Analytical Quality Control, J. Assoc. Publ. Analysts, 1992, **28**, 11-16.

Fig. 1: Diagram of the filter apparatus used in the trial.



## APPENDIX 1

## Analytical Quality Control

General principles of analytical quality control are outlined in protocol V.0 of the series[2].

### 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). For instance, at levels of insoluble matter corresponding to sample C (i.e. 5 mg of insoluble matter in a 5 g sample, or 0.1% *m/m*), r may be taken as 2 mg; this corresponds to a relative standard deviation of repeatability (coefficient of variance of repeatability), $RSD_r$, of 15%. This precision may be expected to deteriorate at lower levels, and the method is suitable as a statutory procedure only when samples with concentrations of insoluble matter in excess of 0.05% *m/m* are analysed.

### 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 (Table 1). For instance, at levels of insoluble matter corresponding to sample C (i.e. 5 mg of insoluble matter in a 5 g sample, or 0.1% *m/m*), R may be taken to be 2 mg; this corresponds to a relative standard deviation (coefficient of variance), $RSD_R$ of 16%. This precision may be expected to deteriorate at lower levels.

### A3. Trueness (Bias)

The observed levels of insoluble matter obtained during the collaborative trial were lower than the expected values; at 5 mg of insoluble matter (0.1% *m/m*), the recovery was 85%, and over 90% at higher levels. There may therefore be a systematic bias, perhaps due to the loss of insoluble material adhering to the filter sieve clamping apparatus.

### 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 insoluble matter content of roughly 0.5 mg of insoluble matter (0.01% *m/m*) for a single determination.

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

Participants in the collaborative trial each analysed ten samples of instant coffee once (five samples in duplicate). Each sample weighed about 5 g, and the whole of it was taken for analysis. Sample A was instant coffee; samples B- E were spiked with known weights of insoluble matter (see Table 1, 'Expected "true" value'). This added insoluble matter consisted of coffee grounds that had been exhaustively extracted with boiling water, filtered and dried.

Table 1 summarises the statistical data; the levels of analyte are expressed as mg of insoluble matter per 5 g sample.

**TABLE 1**

**The Statistical Analysis of Insoluble Matter (mg/5g) in Instant Coffee Samples**

| Sample | A | B | C | D | E |
|---|---|---|---|---|---|
| Number of Laboratories retained after eliminating outliers | 14 | 15 | 16 | 15 | 16 |
| Number of Laboratories eliminated as outliers | 2 | 1 | 0 | 1 | 0 |
| Number of results accepted after eliminating outliers | 28 | 30 | 31 | 30 | 32 |
| LEVEL OF ANALYTE | | | | | |
| Mean observed value $\bar{x}$ | 0.28 | 1.04 | 4.53 | 9.45 | 28.67 |
| Expected "True" Value | A | 1.0+A | 5.0+A | 10.0+A | 30.0+A |
| Recovery (100% = quantitative) (%) | | 76 | 85 | 92 | 95 |
| REPEATABILITY | | | | | |
| Standard Deviation $S_r$ | 0.18 | 0.25 | 0.69 | 0.70 | 0.89 |
| Relative Standard Deviation $RSD_r$(%) | 62 | 24 | 15 | 7.4 | 3.1 |
| Repeatability r [2.8 × $S_r$] | 0.49 | 0.70 | 1.93 | 1.95 | 2.50 |
| REPRODUCIBILITY | | | | | |
| Standard Deviation $S_R$ | 0.25 | 0.32 | 0.72 | 1.01 | 1.34 |
| Relative Standard Deviation $RSD_R$(%) | 89 | 31 | 16 | 11 | 4.7 |
| Reproducibility R [2.8 × $S_R$] | 0.70 | 0.90 | 2.03 | 2.84 | 3.76 |

## 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 |