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## Methods for assessing quality characteristics of non-grain starch staples. (Part 3. Laboratory Methods.)

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# *Methods for Assessing Quality Characteristics of Non-Grain Starch Staples*



PART 3.  
LABORATORY METHODS

# Methods for Assessing Quality Characteristics of Non-Grain Starch Staples

## Part 3. Laboratory Methods

Editors: Z. Bainbridge, K. Tomlins,  
K. Wellings and A. Westby



Overseas Development Administration

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## Section 3.1 Physical analysis

### INSECT-DERIVED WEIGHT LOSS

#### **Introduction**

In many parts of the tropics, cassava is stored as chips which are attacked by a wide range of insect pests. Loss assessment enables an evaluation of the physical losses.

#### *Rationale*

Loss assessment is a useful tool for evaluating the impact of insect pests in stored cassava and the consequent effects on food security. It can also be used for direct comparisons between different systems (e.g. storage techniques or insecticide treatments) and to measure the relative merits of each.

#### *Suitability*

A rapid method of loss assessment which allows a working estimate of losses to be established has been detailed elsewhere (see Section 2.5). In some circumstances, a more precise measure of loss is required, for example, when calibrating the visual scale used in the rapid loss technique, or in studies on differences between insect biotypes. Under these circumstances, there is no alternative to precise determination of loss.

#### *Limitations*

This method is only suitable for well-appointed laboratories and would only be necessary when an absolute measure is required. Otherwise the rapid loss assessment technique should be employed.

#### **Requirements**

##### *Equipment*

- Precision balance
- Oven at 105 °C with drying trays
- Thermometer (reading to at least 120 °C)

#### **Procedure**

The simplest technique is weigh-in and weigh-out (Boxall, 1986) which has been used successfully for cassava chips by Wright *et al.* (1993) in Togo. Weigh the produce or sample at time zero and then reweigh at the time of sample evaluation.

If there has been a change in moisture content (MC) of the cassava chips between the two weighing times, this must be taken into account. To determine the MC of cassava chips, take a representative sample and break into smaller pieces so that, in cross section, they measure a maximum of 3 x 3 cm. Weigh this sub-sample (of the whole amount being monitored) and record the data. Place the sample on an oven tray and place in an oven at 105 °C for 24 h. After this period, reweigh the sub-sample and record the data.

## Analysis of results

### Data record

**Table 3.1** Example of data record table for insect-derived weight loss

Sample	W <sub>o</sub> -Weight of sample at time zero (g)	Dry matter at time zero (%)	Corrected weight at time zero (g)	W <sub>n</sub> -Weight of sample at time = n (g)	Dry matter at time = n (%)	Corrected weight at time = n (g)	Weight loss (%)
KC1	110.00	85	93.50	74.69	83	61.99	33.70

### Calculation

Determine the percentage of weight loss using Equation 1.

#### Equation 1

$$\frac{W_o - W_n}{W_o} \times 100 = \% \text{ weight loss}$$

where: W<sub>o</sub> = weight of whole sample at time zero;  
W<sub>n</sub> = weight of whole sample at time of sample evaluation.

In this form, the method assumes that there has been no change in MC of the cassava chips, as would be the case if they were kept under controlled conditions of temperature and humidity. If there has been any change in MC, chip weight must be adjusted accordingly.

The MC is also given by Equation 1, with W<sub>o</sub> being the weight before drying, W<sub>n</sub> being the weight after drying and the term weight loss being substituted by moisture content.

Having established the MC, correct the weight of the main sample to its dry matter weight, i.e., the weight it would have with an MC of zero. Dry matter is derived using Equation 2.

#### Equation 2

$$DM = \frac{100 - MC \times FW}{100}$$

where: DM = dry matter;  
FW = fresh weight.

The values for dry matter derived from Equation 2 should be used in Equation 1 to determine the true percentage weight loss.

### Significance

The result obtained shows the percentage of weight loss of the sample. How well this reflects the true weight loss in the sample will depend on the uniformity of moisture content and how well the sub-sample used for MC determination reflects any differences.

## International Standards

None published.

## References

BOXALL, R. A. (1986) *A Critical Review of the Methodology for Assessing Farm-Level Grain Losses After Harvest*. Tropical Development and Research Institute (now NRI, Chatham), Report G191. Chatham, UK: Natural Resources Institute.

## Section 3.2 Chemical composition

### CYANOGENS—PYRIDINE/PYRAZOLONE METHOD

#### Introduction

Cyanogens in cassava consist of the following:

- cyanogenic glucosides;
- cyanohydrins (intermediate);
- hydrogen cyanide (HCN, free cyanide).

The method described enables the quantitative determination of each of the three types of cyanogen in fresh roots and processed products.

#### Rationale

Cyanogens are potentially toxic, hence the need to determine accurately their levels in fresh roots and processed products. The need for this is emphasized by the considerable varietal difference in cyanogenic potential and efficiency of the diverse range of processing techniques used traditionally.

#### Suitability

The method allows the accurate determination of cyanogens in fresh cassava root and processed products (including root peel, and fresh and processed cassava leaves).

#### Limitations

The precision of the assay is limited when levels below 5 mg HCN equivalent/kg dry weight are being measured. HCN determination is subject to inaccuracy because of the instability of HCN during the preparation and storage of sample extracts. The nature of the chemicals used in this method necessitates a chemical fume hood or cabinet.

#### Principles

The method enables quantification of HCN by a colorimetric reaction using pyridine/pyrazolone as reagents. The cyanogens are extracted from the roots into a medium containing acid to stabilize the cyanogens, and ethanol to improve the clarification of extracts of gelatinized products, and to prevent microbial growth. In order to determine the cyanogenic potential of the root or processed product, the glucosidic and intermediate cyanogens must be converted to cyanide. This is achieved by enzymatic hydrolysis of cyanogenic glucosides and the manipulation of pH within the assay system in order to convert cyanohydrins into HCN which can then be determined quantitatively (Cooke, 1979; O'Brien *et al.*, 1991). The procedure for the preparation of the linamarase enzyme required in the assay is described.

#### Requirements

##### Equipment

- Freezer for sample storage at -20 °C
- Refrigerator for extracted sample storage
- Waring blender
- Sharp knife
- Buchner apparatus or centrifuge
- VIS Spectrophotometer to measure at 620 nm
- Quickfit test-tubes and stoppers
- Measuring cylinders, 250 ml
- Adjustable-volume pipettes—200 µl, 1000 µl, 5000 µl



## CYANOGENS—PYRIDINE/PYRAZOLINE METHOD

- pH meter and electrode accurate to one decimal place
- Analytical balance accurate to four decimal places
- Water-bath at 30 °C
- Timer
- Spatula/spoon

### *Consumables*

- GF/A filter papers
- Chemical-resistant gloves
- Labels and marker pens
- Storage bottles/vials
- Ethanol, 99% or 96%
- Orthophosphoric acid
- Sodium phosphate
- Sodium hydroxide
- Bispyrazolone (GPR)
- 3-methyl-1-phenyl-5-pyrazolone (GPR)
- Chloramine T (GPR)
- Pyridine (GPR)
- Linamarase (EC 3.2.1.21) BDH or prepared as described in *Associated methods* below
- Potassium cyanide
- pH standards

### *Hazardous chemicals*

<i>Bispyrazolone:</i>	<i>may be harmful by inhalation, ingestion, or skin absorption; avoid inhalation and contact with eyes.</i>
<i>Chloramine T:</i>	<i>may be harmful by inhalation, ingestion, or skin absorption; avoid inhalation and contact with eyes and clothing.</i>
<i>Ethanol:</i>	<i>highly flammable; avoid breathing vapour in high concentrations.</i>
<i>3-methyl-1-phenyl-5-pyrazolone:</i>	<i>may be harmful by inhalation, ingestion, or skin absorption; causes eye and skin irritation; avoid inhalation and contact with eyes and clothing.</i>
<i>Orthophosphoric acid:</i>	<i>causes burns; avoid contact with eyes and skin.</i>
<i>Potassium cyanide:</i>	<i>toxic; serious risk of poisoning by inhalation, swallowing or skin contact; contact with acids liberates a potentially fatal gas, HCN; use in a fume hood.</i>
<i>Pyridine:</i>	<i>highly flammable; harmful vapour; irritating to eyes, skin and respiratory system; use in a fume hood only; avoid inhalation of vapour; avoid contact with skin and eyes.</i>
<i>Sodium phosphate:</i>	<i>may be harmful by inhalation, ingestion, or skin absorption; avoid inhalation and contact with eyes and clothing.</i>
<i>Sodium hydroxide:</i>	<i>causes severe burns; avoid contact with eyes and skin.</i>

## **Procedure**

### *Cyanogen extraction*

*Preparation of extraction media:* add 6.74 ml of orthophosphoric acid (H<sub>3</sub>PO<sub>4</sub>) and 250 ml of ethanol to distilled water; make up to 1 l.

*Fresh roots:* peel roots and remove a longitudinal quarter or half (dependent on size of root). Chop the segment into 1 cm cubes and randomize before sampling. Homogenize 50 g in 180 ml of extraction medium (0.1 M  $\text{H}_3\text{PO}_4$  containing 25% v/v ethanol) for 10 s at low speed followed by 2 x 1 min at high speed with a 1 min rest in between. Use a further 20 ml of extraction medium to wash the homogenate onto a glass fibre GF/A filter. Collect the extract under vacuum using a Buchner apparatus. Store extracts for up to 2 months in a refrigerator.

*Processed products:* randomize the sample by mixing or crushing, depending on the physical nature of the product. Homogenize 30 g of the product for 2 x 1 min in the extraction medium. Filter as described for fresh roots. For extracts of some processed products (especially those that have been heat treated, e.g. gari), clarify by centrifuging at 16 000 *g* prior to filtration. Store extracts for up to 2 months in a refrigerator. Extracts containing peel or from cassava leaves are subject to reduced storability; analyse within 1 week.

## Assay procedure

### Preparation of reagents

- Chloramine T: dissolve 1 g in 200 ml of distilled water in a volumetric flask. Prepare freshly on a daily basis.
- Pyridine/pyrazolone colour reagent: weigh out accurately 0.2 g of bispyrazolone and 1 g of 3-methyl-1-phenyl-5-pyrazolone and dissolve in 200 ml of pyridine; transfer to a dispenser for volumes up to 1 ml; store at room temperature for up to 5 days.
- Linamarase enzyme: dissolve in pH 6 buffer to give an activity of 5 EU/ml; store refrigerated or frozen and check the activity prior to use.
- Sodium hydroxide: for 5 M solution dissolve 200 g in 1 l of distilled water; from this prepare 0.2 M NaOH by taking 40 ml of the stock and making up to 1 l.
- Orthophosphoric acid and buffers: prepare 0.1 M orthophosphoric acid by adding 6.74 ml to 1 l of distilled water; prepare 0.1 M tri-sodium phosphate by dissolving 38 g in 1 l of distilled water; prepare the phosphate buffers at pH 4, 6 and 7 by mixing the above solutions.

*Assay procedure* Cyanogens are assayed in duplicate using the following procedure; see below for a flow chart.

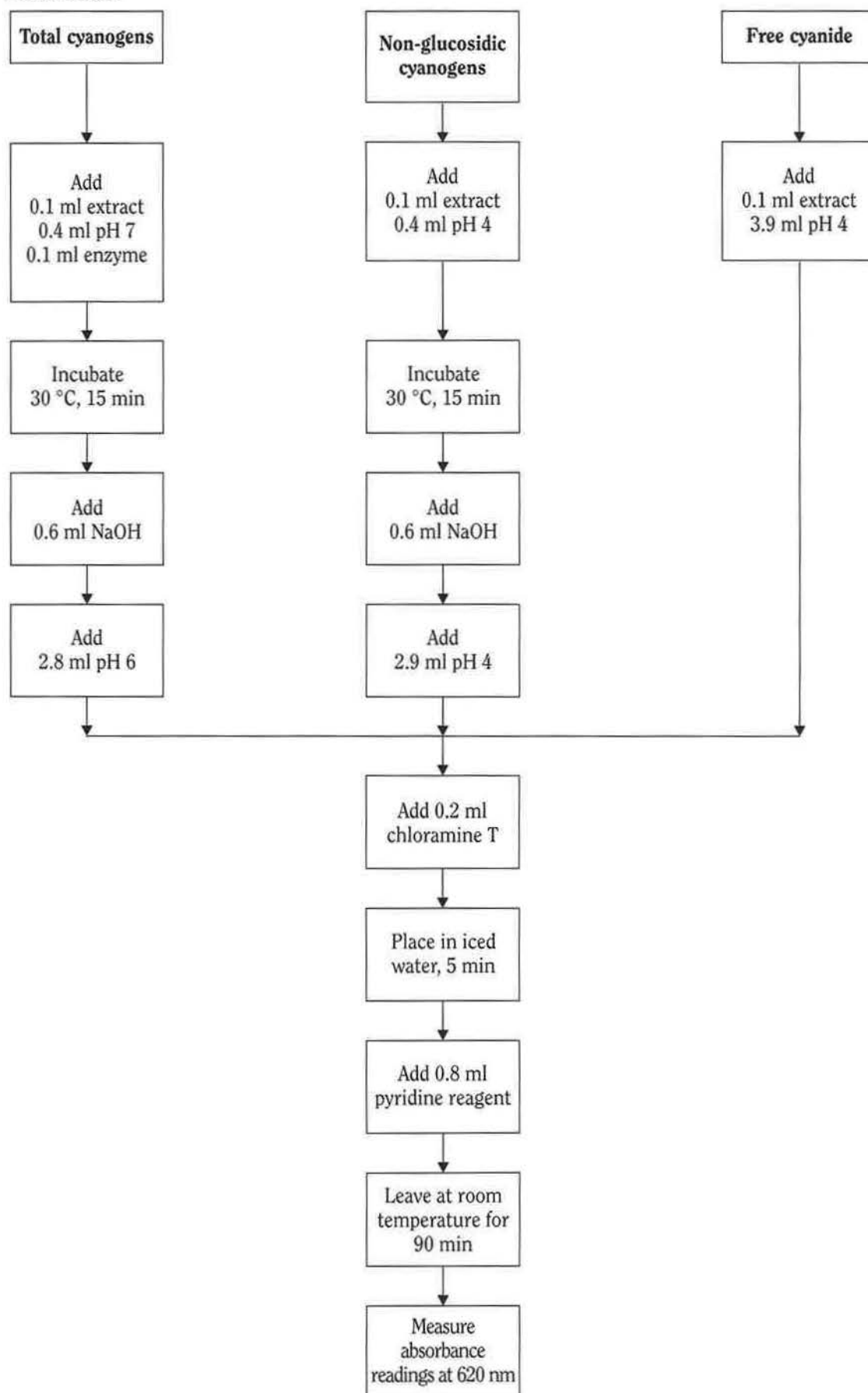
- *Total cyanogens*, i.e., glucosidic, cyanohydrins and HCN: add 0.1 ml aliquots of sample extract to 0.4 ml of pH 7 phosphate buffer in a stoppered Quickfit test-tube. Add 0.1 ml of linamarase enzyme and incubate for 15 min at 30 °C; add 0.6 ml of 0.2 M NaOH, leave for 5 min and add 2.8 ml of phosphate buffer pH 6. Then follow the colorimetric procedure described below.
- *Non-glucosidic cyanogens*, i.e., cyanohydrins and HCN: add 0.1 ml aliquot of extract to 0.4 ml pH 4 phosphate buffer, followed by 0.6 ml of 0.2 M NaOH. After 5 min add a further 2.9 ml of pH 4 phosphate buffer and then follow the colorimetric procedure.
- *Free cyanide*, i.e., HCN: add 0.1 ml aliquot of extract to 3.9 ml of pH 4 phosphate buffer and then follow the colorimetric procedure.

*Colorimetric procedure* Add 0.2 ml of a 0.5% w/v of chloramine T in Quickfit test-tubes to the 4 ml of buffered extract. Mix and place the tubes in an iced water bath for 5 min. In a fume cupboard, add 0.8 ml of the pyridine/pyrazolone reagent. Leave for approximately 90 min; determine the absorbance at 620 nm.

Blanks contain extraction medium in place of sample and are run for each cyanogenic component.

*Important points* Some samples may require dilution prior to analysis for total and non-glucosidic cyanogens; absorbance values ideally lie between 0.1 and 1.0 absorbance units. For samples of low concentration, preparation of a more concentrated sample extract is necessary. Changes to the amount of sample added to the assay system should be avoided because of the sensitivity of the pH system. The activity of the linamarase must be continually monitored. An activity greater than 3 EU/ml is required for the assay (refer to *Associated methods* below).

*Flow chart*



## Standards

Determine levels of cyanogenic content by direct comparison with a known amount of potassium cyanide (KCN) as standard.

With extreme caution, dry KCN over concentrated sulphuric acid for at least 12 h before quantitative preparation of the stock solution. Dissolve 125 mg KCN in 500 ml of 0.2 M NaOH, store, and use for up to 1 month.

Immediately before use, dilute the stock solution 1 in 100 using pH 6 phosphate buffer (use this standard immediately). Run one standard concentration for each of the cyanogenic determinations in duplicate; carry out this procedure daily. Use the following quantities for the standard tubes:

*Blank* Add 0.4 ml pH 7 phosphate buffer, 2.9 ml pH 6 phosphate buffer, 0.1 ml extraction medium and 0.6 ml 0.2 M NaOH; then follow the colorimetric procedure (see below).

*Total standard* Add 0.4 ml pH 7, 2.3 ml pH 6, 0.1 ml extraction medium, 0.6 ml 0.2 M NaOH and 0.6 ml KCN standard solution then follow the colorimetric procedure.

*Non-glucosidic standard* Add 2.7 ml pH 4, 0.1 ml extraction medium, 0.6 ml 0.2 M NaOH and 0.6 ml KCN standard solution; then follow the colorimetric procedure.

*Free standard* Add 3.3 ml pH 4, 0.1 ml extraction medium and 0.6 ml standard solution; then follow the colorimetric procedure.

## Analysis of results

### Data record tables

**Table 3.2** Examples of data record tables for the calculation of cyanogens

Sample	Fresh weight (g)	Moisture content (%)	V'	Corrected average absorbance*		
				Free	I/F	Total
DE1	30.08	16.31	204.91	0.045	0.093	1.650

Sample	Cyanogens (mg HCN equivalent/kg dry weight)			Cyanogens (mg HCN equivalent/kg dry weight)		
	Free	I/F	Total	HCN	Cyanohydrin	Linamarin
DE1	4.16	8.28	144.60	4.16	4.12	136.32

**Note** \*average the assay of the absorbance values, correct by subtracting the blank value, and adjust for any dilution of the original extract.

### Calculation of cyanogens

Use the calculations below:

$$\text{Cyanide (mg HCN}_{\text{equiv.}}/\text{kg) dry weight basis} = \frac{10 \times V' \times A_{620}}{A_{\text{equiv.}} \times \text{DW}}$$

$$V' = V + \frac{[M \times \text{FW}]}{100}$$

where: V = volume of extraction medium (ml);  
 V' = volume adjusted to include sample moisture (ml);  
 A<sub>620</sub> = mean absorbance recorded minus blank;  
 A<sub>equiv.</sub> = absorbance corresponding to 1 µg HCN;  
 DW = dry weight (g);

## CYANOGENS—PYRIDINE/PYRAZOLINE METHOD

- M = moisture of the sample (%);  
FW = fresh weight of sample (g).

To quantify the amount of individual cyanogen components use the following calculation:

- cyanogenic glucoside is total minus non-glucosidic determination;
- cyanohydrins are non-glucosidic minus free cyanogen determination;
- free cyanide is determined by direct measurement.

### Significance

For fresh root, values of total cyanogenic potential range from approximately 50 to 1500 mg HCN<sub>equiv.</sub>/kg on a dry weight basis. As a rough guide to acute toxicity in fresh roots the following guidelines were published by Coursey in 1973:

innocuous: less than 50 mg HCN<sub>equiv.</sub>/kg fresh peeled root;  
moderately poisonous: 50–100 mg HCN<sub>equiv.</sub>/kg fresh peeled root;  
dangerously poisonous: over 100 mg HCN<sub>equiv.</sub>/kg fresh peeled root.

For processed products, the cyanogen content varies greatly.

### International standards

Processing of the roots reduces the cyanogen levels present. International standards for cassava processed products include:

cassava flour: 10 mg HCN<sub>equiv.</sub>/kg dry weight;  
gari: 2 mg HCN<sub>equiv.</sub>/kg dry weight.

**Source:** Codex Alimentarius. CODEX STAN 151–1985 African Regional Standard.

### References

#### Sources

COOKE, R. D. (1978) An enzymatic assay for the total cyanide content of cassava (*Manihot esculenta* Crantz). *Journal of the Science of Food and Agriculture*, **29**: 345–352.

COURSEY, D. G. (1973) Cassava as food: toxicity and technology. *Monograph IDRC-010c*, 27–36.

O'BRIEN, G. M., TAYLOR, A. J. and POULTER, N. H. (1991) Improved enzymic assay for cyanogens in fresh and processed cassava. *Journal of the Science of Food and Agriculture*, **56**: 277–289.

#### Sources for alternative methods

##### Colorimetric methods

The following refers to the most commonly used alternative methods.

NAMBISAN, B. and SUNDARESAN, S. (1984) Spectrophotometric determination of cyanoglucosides in cassava. *Journal of the Association of Analytical Chemists*, **67**: 641–643.

##### Other methods

ASSOCIATION OF ANALYTICAL CHEMISTS (AOAC) (1984) *Official Methods of Analysis*. Williams, S. (ed.) Arlington. Virginia, USA: AOAC Inc.

### Associated methods: linamarase preparation and activity determination

#### Requirements

- High-speed centrifuge
- Centrifuge pots
- Magnetic stirrer and bar
- Dialysis tubing

- Cassava peel
- Acetic acid GPR
- Ammonium sulphate GPR

### *Hazardous chemicals*

*Acetic acid: flammable; causes burns; avoid breathing vapour; avoid contact with eyes and skin.*

### *Preparation of linamarase*

Either purchase the linamarase enzyme (3.2.1.21) required for the determination of total cyanogens from BDH Ltd, Broom Road, Poole, Dorset, BH12 4NN, UK, or prepare from cassava peel as given below.

Chop the fresh cassava peel and homogenize 200 g in batches, i.e., 25 g peel in 200 ml 0.1 M acetate in a blender for 3 min. Centrifuge the homogenate at 10 000 *g* for 30 min and bring the supernatant liquid to about 60% saturation of ammonium sulphate (724 g in 1600 ml) by means of slow addition of the salt and continual gentle agitation of the homogenate. Collect the precipitate by centrifugation at 10 000 *g* for 1 h. Discard the supernatant and dissolve the pellet in 150 ml pH 6 phosphate buffer. Transfer the sample to the dialysis tubing (prepared by boiling in water) and dialyse against pH 6 phosphate buffer (3 x 1.5 l). Aliquot and store frozen.

*Important point:* Ascertain the enzyme activity prior to use in the assay (see below).

### *Testing for exogenous linamarase activity*

Check the activity of the enzyme preparation (see above) or commercially prepared linamarase regularly prior to use in the assay of total cyanogens. A minimum activity of 3 EU/ml (an enzyme unit refers to 1  $\mu\text{mol}$  HCN produced/min) is required.

Dilute the enzyme preparation to 1 in 500 with pH 6 phosphate buffer. Add aliquots of 0.1 ml to tubes containing 0.5 ml of 5 mmol linamarin, in duplicate. Incubate the tubes for 30 min at 30 °C. Stop the reaction by adding 0.6 ml of 0.2 M NaOH. Continue with the colorimetric procedure. Run blanks simultaneously. Add 0.1 ml aliquot of the diluted enzyme preparation to 0.4 ml pH 4 phosphate buffer, followed by 0.6 ml of 0.2 M NaOH. After 5 min, add a further 2.9 ml of pH 4 phosphate buffer and continue with the colorimetric procedure.

Run standards and standard blanks as follows. For blanks, add 0.4 ml pH 7 phosphate buffer, 2.9 ml pH 6 phosphate buffer, 0.1 ml extraction medium and 0.6 ml 0.2 M NaOH, and continue with the colorimetric procedure. For standards, add 2.7 ml pH 4, 0.1 ml extraction medium, 0.6 ml 0.2 M NaOH and 0.6 ml KCN standard solution; continue with the colorimetric procedure.

Calculate the enzyme activity by determining the amount of HCN present in  $\mu\text{mol}$  HCN/min/ml or EU/ml (see *Calculation of enzyme activity* below).

### *Testing for endogenous linamarase activity*

Prepare sample extracts by extracting 30–50 g of sample as described in *Cyanogen extraction* above using pH 6 phosphate buffer instead of 0.1 M  $\text{H}_3\text{PO}_4$ . Dilute the sample extracts as required and analyse as described in *Testing for exogenous linamarase activity* above. Run the appropriate blanks for each extract.

### *Example of the calculation of enzyme activity*

The standard contains 0.605  $\mu\text{g}$  HCN per tube (worked out from the concentration present in the standard solution).

Average absorbance (minus blank) for standard = 0.530, equivalent to 0.605  $\mu\text{g}$  HCN.

Average absorbance (minus blank) for sample = 0.797, equivalent to 0.910  $\mu\text{g}$  HCN.

$$\text{Therefore } \frac{\text{HCN content of sample } (\mu\text{g})}{\text{Molecular weight of HCN}} = \frac{0.910}{27.03} = 0.034 \mu\text{mol HCN produced in 30 min.}$$

$$\text{Therefore } \frac{\text{HCN concentration } (\mu\text{mol})}{\text{Assay time (min)}} = \frac{0.034}{30} = 1.12 \times 10^{-3} \mu\text{mol/min.}$$

To correct for dilution factor of 5000 (i.e., 0.1 ml in assay, 1:500 dilution),

linamarase activity =  $1.12 \times 10^{-3} \times 5000 = 5.6 \mu\text{mol/min/ml}$  or EU/ml.

## CYANOGENS—ISONICOTINATE/1,3-DIMETHYL BARBITURATE METHOD

**Introduction**

Cyanogens in cassava (as previously stated) consist of the following:

- cyanogenic glucosides;
- cyanohydrins (intermediate);
- hydrogen cyanide (HCN, free cyanide).

The method described here enables the quantitative determination of each of the three types of cyanogens in fresh roots and processed products.

*Rationale*

Cyanogens are potentially toxic, hence the need for determining accurately their levels in fresh roots and processed products. This is emphasized by the considerable varietal difference in cyanogenic potential and efficiency of the diverse range of processing techniques used traditionally.

*Suitability*

The method allows the accurate determination of cyanogens in fresh and processed cassava roots and leaves.

*Limitations*

The precision of the assay is limited when measuring levels below 5 mg HCN equivalent/kg dry weight. The determination of HCN already present is subject to inaccuracy because of its instability during preparation and storage of sample extracts. The method has recently been developed and it is recommended that the user performs all controls and standards for comparison.

*Principles*

This method allows the quantification of HCN by means of a colorimetric reaction using isonicotinate/1,3-dimethyl barbiturate as a reagent. In order to determine the cyanogenic potential of the root or processed product, the glucosidic and intermediate cyanogens must be converted to free cyanide. This is achieved by enzymatic hydrolysis of cyanogenic glucosides and the manipulation of pH within the assay system in order to convert cyanohydrins into hydrogen cyanide which can then be quantitatively determined (Essers *et al.*, 1993).

**Requirements***Equipment*

- Freezer for sample storage at -20°C
- Refrigerator for extracted sample storage
- Waring/Braun blender
- Sharp knife
- Table centrifuge up to 6000 *g* plus tubes or Buchner funnel and GF/A filter papers
- Sample bottles
- Measuring cylinders, 250 ml
- Glass beakers, 3 x 500 ml, 2 x 250 ml
- VIS spectrophotometer to measure at 605 nm
- Glass stoppered test-tubes, 7–10 ml
- Adjustable-volume pipettes—200 µl, 1000 µl, 5000 µl and tips
- pH meter and electrode accurate to one decimal place
- Analytical balance accurate to four decimal places
- Water-bath at 30 °C

- Spatula/spoon
- Timer
- Calculator with linear regression or graph paper

### Consumables

- GF/A filter papers
- Chemical-resistant gloves
- Storage bottles/vials, 5 ml
- Ethanol, 96%
- Orthophosphoric acid
- Sodium phosphate
- Sodium hydroxide
- Isonicotinic acid, Fluka 58930
- 1,3-dimethylbarbituric acid, Fluka 59565
- Chloramine T, (GPR)
- Linamarase, (EC 3.2.1.21) BDH 39117
- Linamarin, BDH 40070, stored at -20 °C
- Acetone cyanohydrin, Aldridge A1:000-0
- Potassium cyanide, Merck (96% purity)
- pH standards

### Hazardous chemicals

*Acetone cyanohydrin:* may be fatal if inhaled, swallowed, or absorbed through skin; causes severe irritation; destructive to tissue; use only in a fume cupboard; readily absorbed through skin; avoid contact with skin and eyes.

*Chloramine T:* may be harmful by inhalation, ingestion, or skin absorption; avoid inhalation and contact with eyes or clothing.

*1,3-dimethylbarbituric acid:* may be harmful by inhalation, ingestion, or skin absorption; avoid inhalation and contact with eyes or clothing.

*Ethanol:* highly flammable; avoid breathing vapour in high concentrations.

*Isonicotinic acid:* may be harmful by inhalation, ingestion, or skin absorption; causes upper respiratory tract, eye and skin irritation; avoid inhalation and contact with eyes or clothing.

*Linamarin:* may be harmful by inhalation, ingestion, or skin absorption; causes upper respiratory tract, eye and skin irritation; avoid inhalation and contact with eyes or clothing.

*Orthophosphoric acid:* causes burns; avoid contact with eyes and skin.

*Potassium cyanide:* highly toxic; potentially fatal risk of poisoning by inhalation, swallowing or skin contact; contact with acids liberates a highly toxic gas, HCN.

*Sodium phosphate:* may be harmful by inhalation, ingestion, or skin absorption; avoid inhalation and contact with eyes or clothing.

*Sodium hydroxide:* causes severe burns; avoid contact with eyes and skin.

### Procedure

#### Cyanogen extraction

*Preparation of extraction media:* add 6.74 ml of H<sub>3</sub>PO<sub>4</sub> to 1 l of distilled water; replace 250 ml of water with 250 ml of ethanol if required (ethanol can be used to improve the clarity of extracts, especially for cooked or gelatinized products).



*Fresh roots:* peel roots and remove a longitudinal quarter or half (dependent on size of root). Chop the segment into 1 cm cubes and randomize before sampling. Homogenize 50–70 g in 250 ml of extraction medium, 0.1 M H<sub>3</sub>PO<sub>4</sub> (25% v/v ethanol is optional). Mix for 15 s at low speed, followed by 2 x 1 min at high speed with a 1 min rest period in between. Centrifuge for 10 min at 4000 *g*. Retain the supernatant for subsequent analysis. Alternatively, clarify the extract by means of Buchner apparatus and GF/A filter papers. Store extracts cool or frozen for up to 2 months.

*Processed products:* randomize the sample by mixing or crushing, depending on the physical nature of the product. Take 4 g of flour and swirl gently in 25 ml of refrigerated extraction medium in a closed 50 ml sample jar for at least 5 min. For porridges etc., homogenize 50–70 g in 250 ml of extraction medium. Centrifuge as described for fresh roots. For extracts of some processed products (especially those that have been heat treated e.g. gari), higher centrifugal forces may be required. Store extracts cool or frozen for up to 2 months.

## Colorimetric assay

### Preparation of assay reagents

- Sodium hydroxide: for 5 m solution dissolve 200 g in 1 l of distilled water; from this prepare 0.2 M NaOH by taking 40 ml of the stock and making up to 1 l.
- Orthophosphoric acid and buffers: 0.1 M orthophosphoric acid is prepared by adding 6.74 ml to 1 l of distilled water; 0.1 M tri-sodium phosphate is prepared by dissolving 38 g in 1 l of distilled water; the phosphate buffers at pH 6 and 7 can be prepared by mixing the above solutions.
- Chloramine T: dissolve 0.5 g in 25 ml of distilled water; prepare freshly on a daily basis.
- Colour reagent: dissolve 3.7 g of NaOH in 200 ml of distilled water; add 7.0 g of 1,3-dimethylbarbituric acid and 5.7 g isonicotinic acid and stir extensively; adjust the pH to between 7 and 8 with 1 M HCl or NaOH; storable at room temperature for up to 12 days.
- Linamarase enzyme: dissolve in pH 6 to give an activity of 5 µmol/min/ml; store refrigerated or frozen and check the activity prior to use.

*Assay procedure* Cyanogens are assayed in duplicate using the following procedure for a method flow chart (see below).

- *Total cyanogens*, i.e., glucosidic, cyanohydrins and HCN: add 0.1 ml aliquots of sample extract to 0.4 ml of pH 7 phosphate buffer in a stoppered test-tube. Add 0.1 ml of linamarase enzyme and incubate for 15 min at 30 °C; add 0.6 ml of 0.2 M NaOH, leave for 5 min and add 2.8 ml of phosphate buffer pH 6. Continue, using the colorimetric procedure described below.
- *Non-glucosidic cyanogens*, i.e., cyanohydrins and HCN: add 0.1 ml aliquots of extract to 0.6 ml of 0.2 M NaOH; after 2 min add a further 3.3 ml of pH 6 phosphate buffer and then follow the colorimetric procedure.
- *Free cyanide*, i.e., HCN: add 0.6 ml aliquots of extract to 3.4 ml of pH 6 phosphate buffer and then follow the colorimetric procedure.

*Colorimetric procedure* Add 0.1 ml of chloramine T to the 4 ml of buffered extract in the test-tubes, mix and leave for 5 min. Add 0.6 ml of the colour reagent. Leave for about 10 min (between 8 and 30 min is reasonable), then determine the absorbance at 605 nm.

Blanks contain extraction medium in place of sample and are run for each cyanogenic component.

*Important points* Some samples may require dilution before analysis for total and non-glucosidic cyanogens; absorbance values ideally lie between 0.1 and 1.0 absorbance units, or up to 2.0 in modern digital single beam spectrophotometers. Increasing the absorbance is possible by preparation of a more concentrated extract. The activity of the linamarase must be monitored continually. An activity greater than 3 EU/ml is required for the assay (refer to *Cyanogens-pyridine/pyrazolone method*, *Associated methods* above).

When using the crude enzyme preparation (refer to *Cyanogens-pyridine/pyrazolone method*, *Associated methods*, above for preparation procedure), it is necessary to check for any interference caused by using the following method. To 0.1 ml aliquots of a sample extract, add 0.1 ml, 0.2 ml and 0.3 ml of crude enzyme preparation followed by 0.4 ml, 0.3 ml and 0.2 ml, respectively, of buffer pH 7. If a reduction greater than 5% in the calculated amount of total cyanogens is observed, do not use the enzyme batch tested.

**Flow chart and rationale for the total cyanogen assay**

STEP	ACTION	RATIONALE
Step 1	Extract in acid	Linamarase activity is stopped; cyanohydrins are stabilized
Step 2	Buffer the extract (pH 6); add linamarase; incubate	Glucosides are hydrolysed to cyanohydrins and glucose
Step 3	Raise pH to 12 with NaOH	Cyanohydrins are converted to CN <sup>-</sup>
Step 4	Lower pH to 3–7; add chloramine T; and colour reagent	Each CN <sup>-</sup> ion will trigger the formation of a conjugated molecule with resonance at 605 nm
Step 5	Measure absorbance at 605 nm	
Calibration curve	Total cyanogens or cyanogenic potential determined	

Non-glucosidic cyanogens—same procedure except for Step 2.

Free cyanide—same procedure without Steps 2 and 3, while pH has to be kept between 3 and 5.

## Standards

**Preparation of standard stock solutions** Make these solutions up in volumetric flasks:

- for 5 mmol KCN, dissolve 84.8 mg of dry, solid (96% purity) in 250 ml of 0.2 M NaOH;
- for 5 mmol linamarin (99% purity) stock, dissolve 31.3 mg in 25 ml pH 6 phosphate buffer;
- for 5 mmol acetone cyanohydrin (99% purity), dilute 0.577 ml in 25 ml of 0.1 M H<sub>3</sub>PO<sub>4</sub> and from this take 1 ml and make up to 50 ml with H<sub>3</sub>PO<sub>4</sub>. Because of the poor behaviour of the pure compound when being pipetted, weigh the quantity added for accurate calculation of concentration. The refrigerated stocks are storable for several weeks.

**Working standard solutions** For 80 and 320 µmol working standard solutions, take 1.6 ml of each of the standard stock solutions and make up to 100 and 25 ml, respectively, with 0.1 M H<sub>3</sub>PO<sub>4</sub>; the KCN standard must be prepared immediately before use.

**Procedure** For accurate determination, prepare standards of linamarin, acetone cyanohydrin and KCN as described above. Obtain calibration curves from at least two standards in duplicate between 8 and 40 nmol and 0 nmol of cyanogens per tube by replacing the sample extract with the same volume of standard solution for total and non-glucosidic cyanogen assays. For free cyanide, obtain the calibration curve using 0.1 ml of standard plus 0.5 ml 0.1 M orthophosphoric acid in 3.4 ml pH 6 phosphate buffer.

For proximate assays, use KCN only to determine equivalence values for cyanogens. Assay values will provide a calibration curve which may be used in the calculation of cyanogens.

The calibration curves vary. Therefore it is recommended that calibration standards are run on a daily basis and appropriate values in the calculation used.

## Linamarase activity determination

Refer to *Cyanogens-pyrazolone method, Associated methods*, above.

Note: Substitute the appropriate colorimetric steps.

## Analysis of results

### Data record tables for the cassava cyanogens

**Table 3.3** Example of a data record table for the calculation of cyanogens

Sample	Sample weight (g)	Volume of extraction medium (ml)	Volume of extract assayed (ml)	Moisture content (%)	A <sub>605</sub> *	Quantity of cyanogen per tube (nmol)	Cyanogen level (mg CN <sub>equiv.</sub> /kg dry weight)
SE1	70.0	250	0.1	65.0	0.455	18.66**	58.5

**Notes:** \* average the assay absorbance values, correct by subtracting the blank value and adjust for any dilution of the original extract.

\*\* calibration curve was  $y = -0.006 + 0.0247x$  e.g. if  $y = 0.455$ ,  $x$  is  $(0.455 + 0.006) / 0.0247 = 18.66$  nmol.

### Calculation of cyanogens

Use the equations below to calculate cyanogenic content.

Cyanogen levels are calculated in mg CN equivalent/kg sample on a dry weight basis (mg CN<sub>equiv.</sub>/kg dry weight).

$$[\text{CN}] = \frac{x (v + s (m/100))}{S (1 - (m/100)) d} 0.026$$

where:  $s$  = sample weight (g);

$v$  = volume of extraction medium (ml);

$d$  = volume of extract assayed (ml);

$m$  = moisture content (%);

$x$  = quantity of cyanogen (nmol) in the tube.

0.026 is the conversion factor to express results in mg CN equivalents; to express as mg HCN equivalents, use 0.027.

$x$  is calculated from the calibration curve:

$$x = \frac{(A_{605} - a)}{\text{slope}}$$

where: slope and  $a$  (= intercept) are derived graphically, or from the linear regression of the calibration points by means of a calculator.

Derive the values by including point 0 nmol; for values <0.005 or >0.200 the intercept can be omitted.

### Significance

For fresh roots, values of total cyanogenic potential range from approximately 50 to 1500 mg HCN<sub>equiv.</sub>/kg on a dry weight basis. As a rough guide to acute toxicity in fresh roots, the following guidelines were published by Coursey in 1973:

innocuous: less than 50 mg HCN<sub>equiv.</sub>/kg fresh peeled root;

moderately poisonous: 50–100 mg HCN<sub>equiv.</sub>/kg fresh peeled root;

dangerously poisonous: over 100 mg HCN<sub>equiv.</sub>/kg fresh peeled root.

For processed products, the cyanogen content varies greatly from one product to another.

### International standards

International standards for cassava processed products include:

cassava flour: 10 mg HCN<sub>equiv.</sub>/kg

gari: 2 mg HCN<sub>equiv.</sub>/kg

**Source:** Codex Alimentarius. CODEX STAN 151–1985 African Regional Standard.

### References

#### Source

ESSERS, A. J. A., BOSVELD, M., VAN DER GRIFT, R. and VORAGEN, A. G. J. (1993) Studies on the quantification of specific cyanogens in cassava products and introduction of a new chromagen. *Journal of the Science of Food and Agriculture*, **63**: 287–296.

### *Sources for alternative methods*

#### Colorimetric methods

The following reference indicates the most commonly used alternative methods.

NAMBISAN, B. and SUNDARESAN, S. (1984) Spectrophotometric determination of cyanoglucosides in cassava. *Journal of the Association of Analytical Chemists*, **67**: 641- 643.

#### Other methods

ASSOCIATION OF ANALYTICAL CHEMISTS (AOAC) (1984) *Official Methods of Analysis*. WILLIAMS, S. (ed.). Arlington, Virginia, USA: AOAC Inc.

## DRY MATTER

### **Introduction**

#### *Rationale*

Low moisture content of a processed product is a requirement for a long storage life. The moisture content of a fresh root is related to its dry matter content and therefore to the yield obtained from a particular crop or cultivar.

#### *Suitability*

This method requires the facilities of a laboratory in order to obtain an accurate determination of moisture content/dry matter of fresh and processed NGSS. For a field method, refer to *Part 2, Moisture content*.

#### *Limitations*

The standard analytical method for pastes can be time consuming. An alternative procedure is described that has been successfully used on fermented cassava pastes.

#### *Principle*

Material being analysed is dried under standard conditions; the weight loss incurred is then determined quantitatively.

### **Requirements**

#### *Equipment*

- Analytical balance, accurate to four decimal places
- Desiccator
- L-shaped glass rod
- Metal dishes with close fitting lids, preferably of standard size
- Thermostatically controlled oven for temperatures up to 110 °C

#### *Consumables*

- Sand, 40–100 mesh, acid-washed before use

### **Procedure**

#### *Standard method*

Wash dishes and lids in distilled water. Label corresponding lids and dishes. Place dishes and lids in oven at 80 °C until dry. Leave dishes in oven for 30 min at 105 °C. Only handle dishes with tongs. Remove dishes, with the lids on, from the oven using tongs, and place in desiccator for 30 min. Record the weight of the labelled dishes plus lids ( $W_1$ ).

Chop the fresh root sample into small pieces 1 cm<sup>3</sup>. For large pieces of processed product, break into smaller bits where necessary. Place 2 g of the sample in each dish, replace the lids and weigh accurately to three decimal places ( $W_2$ ). Dry samples

## DRY MATTER

at 105 °C for 4 h with lids ajar. Replace lids and place dishes in desiccator for 30 min to cool. Weigh dishes containing the dried sample ( $W_3$ ).

### *Alternative method for moist pastes*

To each moisture content dish add 25 g of sand and a small glass rod (L-shaped), cover and dry for 30 min at 105 °C. Cool in a desiccator for 20 min. Weigh the dish, lid and the contents ( $W_1$ ).

Add 10 g of sample to the dish plus contents, replace the lid and reweigh immediately ( $W_2$ ). Place the dish on a clean sheet of aluminium foil or clean paper and mix the sample and sand using the glass rod; use the foil to collect and return any stray particles. If necessary, a suitable amount of water may be added to facilitate mixing. Leave the rod in the dish, cover with a lid but leave it ajar, and heat in an oven at 105 °C for 4 h or until a constant weight is obtained ( $\leq 1$  mg). Replace the lid and remove the dishes from the oven, cool in a desiccator for 30 min and weigh ( $W_3$ ).

## Analysis of results

### *Data recording*

**Table 3.4** Example of a data record table for dry matter

Sample	Weight dish (g)	Weight dish + wet sample (g)	Weight dish + dry sample (g)	Moisture content (%)	Dry matter (%)
SQ1	10.507	13.432	12.678	25.778	74.22

### *Calculation*

The calculation for moisture content is as follows:

$$\% \text{ moisture content} = \frac{W_2 - W_3}{W_2 - W_1} \times 100$$

where:  $W_1$  = dish weight;

$W_2$  = dish weight + 'wet' sample;

$W_3$  = dish weight + 'dry' sample.

### *Significance*

Fresh roots typically have moisture contents of 60–80%. Processed products vary depending on the processing method used. For a dry processed product to store well, the moisture content should be lower than 12% to avoid fungal contamination.

## International standards

Codex Alimentarius Draft Standard for flour states that the moisture content shall not exceed 13% w/w; for gari, moisture content shall not exceed 12% w/w.

## References

AOAC (1984) Moisture content in flour. 14.004.

SLACK, T. (1987) *Analytical Methods Manual*. 2nd edn. Leatherhead, UK: Leatherhead Food Research Association.

## ETHANOL IN BEVERAGES

### **Introduction**

NGSS are used as substrates throughout the brewing industry, from traditional home processing of beer for local consumption to industrial-scale production of spirits. The following method allows the determination of the ethanol content in the final liquid product.

### *Rationale*

The ethanol content is a key parameter for indicating the quality of the product whether it is for home consumption as a beverage or for industrial use.

### *Suitability*

The method described can be used for the determination of ethanol in a wide variety of alcoholic beverages and spirits.

### *Limitations*

The titration used in this procedure is very accurate and has a sharp end-point; the main essential is to use *absolutely clean* glassware, free from grease or any organic material which might be oxidized by dichromate. Volatile components in the sample other than ethanol that can be oxidized by dichromate may interfere with the results.

### *Principle*

Alcohol is distilled from the sample and collected in an acid solution of potassium dichromate where it is oxidized to acetic acid at 60 °C. The residual dichromate is determined by back titration with ferrous sulphate in a strongly acid solution, using ferroin indicator (1,10-phenanthroline-ferrous sulphate complex).

A similar titration is used in the determination of Chemical Oxidation Demand (COD) in effluent evaluation.

## **Requirements**

### *Equipment*

- Quickfit condenser, Inland Revenue with 19/26 cone and socket joints C6/12/SC
- Quickfit splash head, vertical with 24/29 and 19/26 cone joints SH7/13
- Quickfit straight delivery adapter with 19/26 socket RA1/22
- Quickfit 500 ml flask, round-bottomed, uniform height of 205 mm with 70 mm neck and 24/29 socket FR500/3U
- Quickfit 150 ml conical flask with 24/29 socket FE150/3
- Quickfit stopper, 24/29 cone
- Heating mantle for 500 ml flask
- 25 ml burette, Grade A
- 25 ml pipettes, bulb type Grade A
- 500 ml, 1 l and 2 l volumetric flasks, Grade A

### *Consumables*

- Ferrous ammonium sulphate
- Ferroin indicator BDH 21056 (1,10-phenanthroline-ferrous sulphate complex solution 0.025 mol/l redox indicator)
- Potassium dichromate, AR
- Sulphuric acid, concentrated
- Anti-bumping granules

### *Hazardous chemicals*

*Ferrous ammonium sulphate:* *may be harmful by inhalation, ingestion or skin absorption; causes eye and skin irritation; avoid contact with eyes and skin.*

*1,10-Phenanthroline-ferrous sulphate:* *harmful if swallowed, inhaled or absorbed through skin; may cause irritation; avoid contact with eyes and skin.*

*Potassium dichromate:* *contact with combustible material may cause fire; may be fatal if swallowed, inhaled or absorbed through skin; avoid inhaling the dust; avoid contact with eyes or skin especially if the latter is cut or cracked.*

*Sulphuric acid: causes severe burns; prevent contact with skin and eyes.*

## Procedure

### Preparation of reagents

*Potassium dichromate (0.25 N)* Dissolve 6.129 g of potassium dichromate in water and make up to 500 ml. Keep in an amber glass-stoppered bottle.

*Sulphuric acid (approximately 25 N)* Place 300 ml water in a 2 l conical flask and cautiously add 600 ml of sulphuric acid AR, cooling in a bath of tap-water during addition. Keep in a glass bottle.

*Ferrous ammonium sulphate (approximately 0.1 N)* Dissolve 40 g ferrous ammonium sulphate in water, then add 30 ml of sulphuric acid (concentrated). Cool the solution and then make up to 1 l with water. **Note:** this is best made up as required as the strength slowly diminishes due to oxidation by atmospheric oxygen.

*Ferriin indicator* (1,10-phenanthroline-ferrous sulphate complex solution 0.025 mol/l redox indicator, BDH 21056).

### Method

Add a known volume of sample which contains up to 20 mg of ethanol (the volume required is dependent on ethanol concentration, adjusted accordingly) and anti-bumping granules to 50 ml of distilled water and distil from a 500 ml round-bottomed Quickfit flask. Collect the distillate in a 150 ml Quickfit conical flask marked at 35 ml and containing 10 ml of 0.25 N potassium dichromate (i.e., 25 ml of distillate is collected).

Add 25 ml of 25 N sulphuric acid (using measuring cylinder) to the flask, and promptly stopper it. The volumes are chosen so that the temperature after mixing is  $\approx 60$  °C. Wait 30 min and then titrate the contents of the flask.

Run a blank at the same time. Add 10 ml of 0.25 N potassium dichromate, 25 ml 25 N sulphuric acid and 25 ml water to a 150 ml flask, and cool. Then titrate.

*Titration* Add two drops of ferriin indicator to the contents of the flask, and then titrate 0.1 N ferrous ammonium sulphate from a 25 ml burette. The colour gradually changes from yellow to blue-green, then changes in one drop through grey to pinkish-brown; this is the end-point. Note the volume titrated.

## Analysis of results

### Calculation of ethanol content

$$\text{Ethanol (in mg) per ml of sample} = \frac{1.152 \times (B-T) \times 25/B}{\text{sample volume (ml)}}$$

where: T = ml titration of sample;

B = ml titration of blank.

## Example

A solution of absolute alcohol was made up which contained, by weight, 7.86 mg/ml.

**Table 3.5** Example of a typical titration using the 7.86 mg/ml concentration

Volume of ethanol solution (ml)	Titration (ml)	Blank-titration B-T (ml)	Quantity of ethanol present (mg)	
			Total	per ml
Blank 0 ml	25.15	0.00	—	—
1	18.22	6.93	7.94	7.94
2	11.30	13.85	15.86	7.93
3	4.47	20.68	23.68	7.89

## International standards

None known.

## References

- CAPUTI, A. and WRIGHT, D. (1969) Collaborative study of the determination of ethanol in wine by chemical oxidation. *Journal of the Association of Official Analytical Chemists*, **52**(1): 85–88.
- JOLLY, S. C. (ed.) (1963) Determination of Chemical Oxidation Demand (COD) in effluent evaluation. pp. 175–177. In: *Official, Standardised and Recommended Methods of Analysis*. Cambridge, UK: W. Heffer & Sons Ltd.

## TANNINS

### Introduction

Tannins are complex phenolic polymers which are classified into two structural groups: hydrolysable tannins and condensed tannins. The latter are far more common in NGSSs and usually exist in the plant tissues as leucoanthocyanins or proanthocyanidins. These condensed tannins have been positively identified in cassava roots, in the green pulp of many banana varieties and in yam (*Dioscorea*) species. Their presence is suspected in *Colocasia* and *Xanthosoma* spp.

### Rationale

Tannins bind to both proteins and carbohydrates; this has several implications for commodities containing tannins. The presence of tannins can cause browning or other pigmentation problems in both fresh food and processed products. Tannins can also act as anti-nutritionals in two ways. Firstly, they can provoke an astringent reaction in the mouth and make the food unpalatable. Secondly, they can form complexes with and thus precipitate proteins in the gut, reducing digestibility or inhibiting digestive enzymes and micro-organisms. This has implications for both human and livestock nutrition.

### Suitability

The assay for condensed tannins described below was developed to compare tannin levels in the green pulp of different banana varieties. A modification of this method has been used with fresh and processed cassava (Rickard, 1986). The suitability of the method for measuring tannins from other NGSSs is not known.

### Limitations

The size and complexity of tannin molecules, and their capacity for binding to proteins and carbohydrates, can make them exceedingly difficult to extract from and assay in plant tissues. This is the primary limitation of all methods to measure tannins in plant tissues. For this reason, some researchers have added the reagent directly to the dried plant tissues rather than extracting the tannins with solvent beforehand (Rickard, 1986). Another limitation is the lack of suitable tannin standards. This means that measurements are relative, not absolute.

### Principle

The method allows the relative measurements of condensed tannins by means of condensation of the tannin with vanillin to a coloured compound which can be measured using a colorimeter or spectrophotometer (Broadhurst and Jones, 1978).

## Requirements

### Equipment

- Freeze drier
- Coffee grinder
- Balance
- Magnetic stirrer and 'flea'
- Buchner funnel
- Vacuum pump
- Rotary evaporator or heating block with air
- Water-bath
- Quickfit test-tubes and stoppers



## TANNINS

- Colorimeter or spectrophotometer to measure at 500 nm.

### *Consumables*

- Liquid nitrogen
- Acetone
- Acetic acid
- Aluminium foil
- GF/A filter papers
- Hydrochloric acid
- Vanillin
- Ethanol

### *Hazardous chemicals*

*Acetone:* may be harmful by inhalation, ingestion, or skin absorption; causes irritation to eyes, skin and upper respiratory tract; avoid inhalation of vapour, contact with skin or eyes; extremely flammable; keep away from heat, spark or open flame.

*Ethanol:* may be harmful by inhalation, ingestion, or skin absorption; causes irritation to eyes, skin and upper respiratory tract; avoid inhalation of vapour, contact with skin or eyes; extremely flammable; keep away from heat, spark or open flame.

*Nitrogen:* may be harmful; at high concentrations in the air it acts as an asphyxiant; always wear gloves to protect from cold burns; avoid contact with eyes or skin.

*Vanillin:* may be harmful by inhalation, ingestion, or skin absorption; may cause irritation; avoid inhalation of vapour; avoid contact with skin or eyes.

## **Procedure**

### *Preparation of samples*

Sufficient replication of samples must be considered prior to sample preparation. This is covered in *Part 1, Introduction, Sampling*.

Remove the peel from fresh fruit samples (green or ripe). Cut the pulp into slices about 1 cm thick and plunge them immediately into liquid nitrogen. When the samples have reached equilibrium temperature, they can be held frozen by storage in a freezer, or immediately dried in a freeze drier. In this way, changes in metabolites, including tannins, will be minimized. When dry, grind the samples to a super-fine powder and store in airtight containers until ready for analysis. Do not grind samples more than 48 h before analysis.

### *Tannin extraction*

Place 2 g of freeze-dried ground sample into a 50 ml conical flask and add 30 ml of an acetone:10% acetic acid (80:20 v/v, i.e., the final solvent mixture contains 2% acetic acid). Swirl the powder in the solvent using a magnetic stirrer and 'flea' for 15 min. Allow the pulp to settle at the bottom of the flask; remove the solvent (containing most of the tannins) by suction and filter through a glass fibre (GF/A) filter paper under vacuum using a Buchner funnel. Wash the pulp with two more 10 ml aliquots of solvent and combine the filtered washings. Remove the acetone from the filtrate using a rotary evaporator set at 35 °C.

### *Tannin analysis*

Dilute the extracts 20-fold using distilled water before assaying. Prepare sufficient 15 ml Quickfit test-tubes to exclude light either by painting them black or by covering with aluminium foil. Place 0.5 ml of diluted sample in each test-tube followed by 3 ml of freshly prepared 4% vanillin in ethanol (w/v). The solutions should be mixed thoroughly before and after adding 1.5 ml of concentrated HCl. The presence of condensed tannins is indicated by the development of a deep red colour. Leave the test-tubes at room temperature for 15 min before measuring absorbances of the samples at 500 nm using a spectrophotometer or

colorimeter. Note that the readings are temperature sensitive, so the assay must be performed at the same temperature each time. The assay was originally developed at  $20 \pm 2$  °C; however, preliminary research has indicated that consistent results can be obtained at 30 °C and 35 °C.

### *Preparation of standards*

There are no useful standards for the comparison of tannins in NGSSs. Tannic acid, (+) catechin and cyanidin chloride have been used by some researchers to develop standard curves for tannin analysis. However, natural tannins are highly variable and the results of different assays vary considerably, depending on the structure of the monomers making up the tannin and on the degree of polymerization (Price *et al.*, 1978).

### **Analysis of results**

As there are no tannin standards, it is not possible to calculate actual quantities of tannin with any degree of accuracy. A relative scale for a particular commodity type has to be devised in each case. The relative units could be, for example, absorbance unit/mg of sample or, if a standard curve is prepared from for example, (+) catechin, units could be expressed as mg (+) catechin equivalent/100 g dry weight.

### *Significance*

As explained above, measurements of tannins in NGSSs are relative not absolute. Figures quoted in the literature are given in a range of relative units and values are highly variable, depending, in particular, on the assay method used.

### **International standards**

There are no international standards for tannins in NGSSs.

### **References**

BROADHURST, R. B. and JONES, W. T. (1978) Analysis of condensed tannins using acidified vanillin. *Journal of the Science of Food and Agriculture*, **29**: 788–794.

PRICE, M. L., VAN SCOYOC, S. and BUTLER, L. G. (1978) A critical evaluation of the vanillin reaction as an assay for tannin in sorghum grain. *Journal of Agriculture and Food Chemistry*, **26**(5): 1214–1218.

RICKARD, J. E. (1986) Tannin levels in cassava, a comparison of methods of analysis. *Journal of the Science of Food and Agriculture*, **37**: 37–42.\*

*The following reference is a critical review of analytical techniques which have been used to estimate tannin content of plant tissues. Different assays can result in widely varying estimates of tannin content since they are designed to measure different characteristics.*

HAGERMAN, A. E. and BUTLER, L. G. (1989) Choosing appropriate methods and standards for assaying tannin. *Journal of Chemical Ecology*, **15**(6): 1795–1810.

## PROXIMATE COMPOSITION

### **Introduction**

Analysis of proximate composition provides information on the basic chemical composition of the NGSS fresh commodity or processed product. Routine methods for protein, fibre, oil, sugars and ash determinations are covered in this section. These components are fundamental to the assessment of the nutritive quality of the food being analysed.

There are several modifications currently being used that allow for improved efficiency and convenience, and that reduce the need for chemical resources. The methods described in this section do not cover all such modifications. However, this does not preclude the use of available equipment and reagents where the performance is unaffected by their use.

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\*The protein precipitate method used in this comparative study is now no longer recommended.

## **Total nitrogen and non-protein nitrogen**

### *Introduction*

#### Rationale

The protein component of NGSS is limiting in some commodities in terms of nutritional value. Screening of varieties and improved processing may result in varieties and processed products of higher nutritive quality. This method can be used routinely by breeders and processors to ascertain protein levels in fresh and processed NGSS.

#### Suitability

These methods allow the accurate determination of total nitrogen, total soluble nitrogen and non-protein nitrogen in a very wide range of fresh and processed products.

#### Limitations

The method described is the most reliable technique for the determination of organic nitrogen and is applicable to almost all fresh and processed products. The only limitation to the method is that the amount of sample which can be analysed depends on its carbohydrate content. This chars and is difficult to digest cleanly.

#### Principles

Although the Kjeldahl procedure has been modified many times, the basic procedure is still the most reliable technique for the determination of organic nitrogen. The method is based on the digestion of the sample with hot concentrated sulphuric acid in the presence of a metallic catalyst. Organic nitrogen in the sample is reduced to ammonia; this is retained in solution as ammonium sulphate. The digest is made alkaline, then distilled to release the ammonia. The ammonia is trapped in dilute acid and titrated.

### *Requirements*

#### Equipment

- Quickfit Macro Kjeldahl Distillation Unit comprising:
  - condenser C1/12/SC;
  - dropping funnel D62/50GP;
  - multiple adapter MA1/3;
  - double spring clips (2) MF40/13/24;
  - delivery adapter RA1/22;
  - splash head SH7/12;
  - 300 ml Kjeldahl flask, 300/3L (1 per sample);
  - 150 ml Erlenmeyer flask with 24/29 socket FE150/3;
  - MSE homogenizer and 100 ml flask (non-protein nitrogen only);
  - burette, 25 ml;
  - Bunsen;
  - microburner.

#### Consumables

- Boric acid
- Copper sulphate
- Selenium dioxide
- Sulphuric acid, AR
- Potassium sulphate
- Hydrochloric acid, 0.1 N

- Sodium hydroxide
- Bromocresol green
- Anti-bumping granules
- Sodium citrate
- Trichloroacetic acid (non-protein nitrogen only)
- Whatman No. 41 filter paper (non-protein nitrogen only)

### *Hazardous chemicals*

<i>Boric acid:</i>	<i>may be harmful by inhalation, ingestion, or skin absorption; may cause irritation; readily absorbed through skin; avoid contact and inhalation.</i>
<i>Bromocresol green:</i>	<i>may be harmful by inhalation, ingestion, or skin absorption; may cause irritation; avoid inhalation and contact with eyes and skin.</i>
<i>Copper sulphate:</i>	<i>harmful if inhaled or swallowed; causes skin and severe eye irritation; avoid contact with eyes and skin; do not inhale dust.</i>
<i>Hydrochloric acid:</i>	<i>may be fatal if inhaled, swallowed or absorbed through skin; causes burns, material is extremely destructive; avoid inhalation of vapour and contact with eyes and skin.</i>
<i>Potassium sulphate:</i>	<i>may be harmful by inhalation, ingestion, or skin absorption; swallowing may cause severe gastrointestinal irritation; avoid inhalation of dust; avoid contact with eyes and skin.</i>
<i>Selenium dioxide:</i>	<i>may be fatal if inhaled, swallowed or absorbed through skin; material is extremely destructive to the upper respiratory tract, eyes and skin; avoid contact with eyes and skin; avoid inhalation of dust.</i>
<i>Sodium hydroxide:</i>	<i>harmful if inhaled, swallowed, or absorbed through skin; material is extremely destructive to the upper respiratory tract, eyes and skin; readily absorbed through skin; avoid contact with skin and eyes; toxic; corrosive.</i>
<i>Sulphuric acid:</i>	<i>may be fatal if swallowed; harmful if inhaled or absorbed through skin; causes burns; material is extremely destructive to upper respiratory tract, eyes and skin; avoid inhalation of vapour; avoid contact with skin, eyes and clothing; highly toxic; corrosive; reacts violently with water.</i>
<i>Trichloroacetic acid (non-protein nitrogen only):</i>	<i>harmful by inhalation, ingestion, or skin absorption; may cause irritation; material is extremely destructive to upper respiratory tract, eyes and skin; avoid inhalation of vapour; avoid contact with skin, eyes and clothing; possible mutagen.</i>

### *Procedure*

#### *Preparation of reagents*

*Kjeldahl catalyst solution* Dissolve 2.5 g of copper (II) sulphate pentahydrate and 2.5 g of selenium dioxide in 250 ml of water (in a 1 litre conical flask). Add slowly, in portions, 250 ml of concentrated sulphuric acid; cool the flask between additions in a bath of running tap-water. Store the blue solution in a glass bottle closed by a polypropylene screw cap.

A number of alternative catalysts have been suggested including a mixture of copper (II) sulphate and titanium dioxide (0.15 g copper (II) sulphate pentahydrate and 0.15 g titanium dioxide/digestion). However, this catalyst is not as effective as selenium-based catalysts.

*Hydrochloric acid 0.05 N* Transfer the contents of one ampoule of 0.1 n standard volumetric solution of hydrochloric acid to a 1 l volumetric flask and make up to 1 l with distilled water.

*Sodium hydroxide solution, approximately 11 N* Dissolve 125 g of sodium hydroxide pellets in 250 ml of water and cool. Keep this solution in a *plastic* bottle.

## TOTAL NITROGEN AND NON-PROTEIN NITROGEN

*Boric acid 2% w/v* Tip 20 g boric acid AR into a 1 l glass bottle and add 1 l of water.

*Bromocresol green* (BCG). Dissolve 250 mg in 250 ml water.

*Methyl red* (MR) Water-soluble. Dissolve 50 mg in 250 ml water.

*Ma and Zuazaga's mixed indicator* Mix equal volumes of BCG and MR solutions. The solution deteriorates after a few days and then does not give a sharp end-point. Alternatively, add an equal number of drops of the two solutions before titration.

*Anti-bumping granules* Bumping can be a problem both in the digestion and in the distillation steps.

*Sodium citrate solution* Prepare a fresh 1% w/v (approximate) solution each time a batch of estimations is run. This prevents the precipitation of copper hydroxide and reduces bumping during the distillation.

### Total nitrogen

*Digestion* *Note:* the amount of sample which can be added depends on the content of carbohydrate, which chars and is difficult to digest cleanly.

To the digestion flasks add 2 g of sample (adjust weight accordingly if excess charring or low levels of protein are observed; ideally use a sample weight containing 0.03–0.04 g N) and a few anti-bumping beads, 2 g potassium sulphate and 10 ml of Kjeldahl catalyst. Wet the ground glass joint of the flask with acid if the flask is to be stoppered and not digested immediately.

Semi-solid materials, such as fresh vegetables, can be conveniently weighed on to a small filter paper, and the filter paper wrapped around the sample and dropped to the bottom of the digestion flask.

Clamp the flask at an angle of 30° to the vertical, *in a fume cupboard*, and heat with a small gas flame. This should allow the sulphuric acid to boil gently towards the end of digestion, and reflux in such a way as to wash down all parts of the flask which may become spattered with organic matter. If there is much charring, turn the flame down once water has boiled off. It may be necessary later on, with high-carbohydrate samples, to remove the flask and tilt it cautiously so as to wash down char from the flask wall. After 1–3 h the solution should be clear and light green or blue.

*Allow the flask to cool*, and then add 50 ml of sodium citrate solution using a cylinder. Cool the flask under the tap and stopper until it is distilled.

*Distillation* Open the flask, add more anti-bumping granules and then connect to the still. Use a 150 ml Quickfit conical flask as receiver, to which add 25 ml of 2% boric acid using a measuring cylinder and 5–10 drops of mixed indicator (or 3–4 drops of MR + 3–4 drops BCG); the colour should now be a purplish-red. Adjust so that the condenser just dips into the boric acid.

Run 25 ml of sodium hydroxide into the tap funnel and cautiously run into the flask. Rock the still gently to mix the contents, which become deep blue. Heat the flask with a micro-burner.

*NOTE:* as long as the condenser dips into the boric acid, heating must be continuous, otherwise the boric acid may be sucked back.

After 5 min lower the receiver and continue the distillation until the potassium sulphate crystallizes out and *violent* bumping commences. Remove the flame at once. Lower the condenser and wash out with a few ml of distilled water.

If the sample contains nitrogen, the ammonia turns the indicator bright green.

*Titration* Using a 25 ml burette, titrate with 0.05 N hydrochloric acid. The indicator colour changes from green to grey to pink within 1–2 drops. The exact tint at the end-point is a matter of preference, and does not greatly matter since a blank is run (for the blank, follow the same procedure omitting the sample).

### Non-protein nitrogen

The determination of total nitrogen by normal Kjeldahl procedures will not include inorganic nitrogen from, for example, nitrates and nitrites. Non-protein nitrogen is high in certain foods such as fish, fruit and vegetables.

Place 2 g of sample into a 100 ml MSE homogenizer flask, and add 50 ml of freshly prepared 10% w/v trichloroacetic acid. Homogenize at full speed for 1 min.

Filter the sample into a 100 ml volumetric flask using a Whatman No. 41 paper. Make up to volume with 10% trichloroacetic acid.

Pipette 20 ml into a Kjeldahl flask and digest as for *Total nitrogen*.

### *Analysis of results*

#### Data recording

**Table 3.6 Example of data record table for total nitrogen, non-protein nitrogen and crude protein**

Total nitrogen					Non-protein nitrogen				Crude protein
Sample	Sample weight (g)	Titration volume (ml)	Titration blank (ml)	Total nitrogen (%)	Sample weight (g)	Titration volume (ml)	Titration blank (ml)	Non-protein nitrogen (%)	Crude protein (%)
JR1	2.00	16.00	1.03	1.05	2.00	5.20	1.20	1.40	6.55

#### Calculation of total nitrogen

$$\% \text{ total nitrogen} = \frac{0.14 \times (\text{titration (ml)} - \text{blank (ml)})}{\text{weight of sample (g)}}$$

#### Calculation of non-protein nitrogen

$$\% \text{ non-protein nitrogen} = \frac{0.14 \times 5 \times (\text{titration (ml)} - \text{blank (ml)})}{\text{weight of sample (g)}}$$

#### Calculation of crude protein

The crude protein content can be obtained by multiplying the total nitrogen content by a factor of 6.25.

### *International standards*

None known.

### *References*

EGAN, H., KIRK, R. and SAWYER, R. (eds) (1981) Nitrogen and crude protein, the Kjeldahl procedure. pp. 15–19. In: *Person's Chemical Analysis of Foods, General Chemical Methods*. 8th edn. Harlow, UK: Longman Scientific and Technical.

## **Neutral detergent fibre (cell walls)**

### *Introduction*

#### Rationale

The neutral detergent procedure for cell walls (CW) described here is a rapid method for the determination of total fibre in fibrous NGSS and processed products. It appears to divide the dry matter of food very near to the point which separates the nutritively available and soluble constituents from those which are incompletely available or dependent upon a microbial fermentation.

#### Suitability

This method is suitable for fresh commodities and processed products.

## NEUTRAL DETERGENT FIBRE (CELL WALLS)

### Limitations

This method cannot be used with fresh and processed products that have a high protein and low fibre content. Where samples have a high ash content, it will not give an accurate determination of fibre.

### Principles

The method provides a simple and rapid means of determining neutral detergent fibre by a gravimetric procedure.

### Requirements

#### Equipment

- Analytical balance accurate to four decimal places
- Hot-plate
- 1 l Quickfit Erlenmeyer flask FE1L/E with 24/29 socket and condenser
- Fritted glass crucibles, coarse porosity (2) – note the cleaning procedure; or Buchner funnel and Whatman 541 filter paper
- Rubber gasket
- 1 l volumetric flask
- 10 ml pipette
- 2 ml pipette
- Timer
- Vacuum pump
- Knife
- Lab-mill/pestle and mortar
- Muffle oven (not essential)
- Sieve, 1 mm
- Glass rod

#### Consumables

- Sodium dodecyl sulphate
- Ethylenediaminetetraacetic acid (EDTA)
- Disodium hydrogen orthophosphate
- Sodium borate decahydrate (borax)
- 2-ethoxyethanol
- Decahydronaphthalene (Decalin)
- Acetone
- Sodium sulphite
- Whatman No. 541 ashless filter paper

#### Hazardous chemicals

##### Acetone:

*may be harmful by inhalation, ingestion, or skin absorption; causes irritation; vapour is irritating to eyes and upper respiratory tract; avoid contact with eyes and skin; highly flammable; keep away from sparks, heat or flame.*

##### Decahydronaphthalene, Decalin:

*may be harmful by inhalation or ingestion; causes irritation; vapour is irritating to eyes and upper respiratory tract; causes skin irritation; avoid inhalation of vapour; avoid contact with eyes and skin; keep away from heat and flame; potentially explosive peroxides may form.*

- Disodium hydrogen orthophosphate:* may be harmful by inhalation, skin absorption or ingestion; causes irritation to eyes and skin; avoid inhalation of dust.
- 2-ethoxyethanol:* harmful by inhalation or ingestion; causes irritation; vapour is irritating to eyes and upper respiratory tract; causes skin irritation; avoid inhalation of vapour and contact with eyes and skin; readily absorbed through skin.
- Ethylenediaminetetraacetic acid (EDTA):* may be harmful by inhalation, ingestion, or skin absorption; causes eye, skin and upper respiratory tract irritation; avoid contact with eyes and skin.
- Sodium dodecyl sulphate:* harmful if inhaled; may be harmful if swallowed or absorbed through skin; causes eye and skin irritation; irritating to upper respiratory tract; use only in a fume cupboard; avoid inhalation and contact with eyes and skin.
- Sodium sulphite:* may be harmful by inhalation or ingestion; causes irritation to eyes and skin; avoid contact with eyes and skin.

## Procedure

### Preparation of neutral detergent solution

Add 30 g of sodium dodecyl sulphate, 18.61 g EDTA, 4.56 g disodium hydrogen orthophosphate, 6.81 g sodium borate decahydrate (borax) and 10 ml 2-ethoxyethanol to 1 l of distilled water and dissolve. The pH should be within the range of 6.9–7.0; adjust using sulphuric acid or sodium hydroxide if necessary.

### Determination of neutral detergent fibre

For samples with a high moisture content, the material may require drying before analysis. Where this applies, it is best to measure the fibre content on a dry weight basis. If the samples are of consistently low moisture content, the drying step may be omitted.

Dry 10 g of sample in order to obtain the dry matter content (refer to *Dry matter* above). Mill the dried material so that it passes through a 1 mm<sup>2</sup> aperture. Accurately weigh 1 g of the sieved sample and transfer to a 250 ml Erlenmeyer flask. Add 0.5 g of sodium sulphite. Add, in order, 100 ml neutral detergent solution, 2 ml Decalin. Heat to boiling in 5–10 min on a hot-plate. Reduce heat as boiling begins, to avoid foaming. Adjust boiling to an even level and reflux for 60 min timed from the onset of boiling.

*NOTE:* Either glass crucibles or a Buchner funnel and Whatman 541 filter papers may be used for filtration of the fibre material.

*Filtration using glass crucibles* Remove container, swirl and filter through pre-weighed fritted glass crucible (alternative filtering apparatus described below) under gentle suction. Shut off vacuum. Break up the filtered mat with a rod and fill the crucible two-thirds full with hot (90–100 °C) water. Stir and allow to soak for 15–30 s. Vacuum dry and repeat the water washing, rinsing the sides of the crucible. Wash similarly with acetone twice. Repeat acetone washings until no more colour is removed, breaking up any lumps so that the solvent wets all the fibre. Remove the residual acetone using a vacuum. Dry at 103 °C overnight, cool in a desiccator and weigh.

*Filtration using Buchner apparatus* Into a Buchner funnel place a Whatman No. 541 filter paper. Pour boiling water into the funnel, allow the water to remain until the funnel is hot and then drain by applying suction. After refluxing the sample for 60 min, allow the mixture to stand for 1 min, then pour into a shallow layer of hot water under gentle suction in the prepared funnel. The filtration of the sample should be complete within 10 min. Wash the insoluble matter twice with boiling water and then wash twice with acetone. Transfer the insoluble matter to a dried, weighed, Whatman No. 541 ashless filter paper and dry overnight at 103 °C.

### Cleaning crucibles

Crucibles tend to become clogged with residual matter after much use. A reliable cleaning procedure is to ash at 500 °C and force water in reverse flow through the filter plate. When crucibles become clogged with mineral particles after several uses, a hot solution of 20% KOH, 5% Na<sub>3</sub>PO<sub>4</sub> and 0.5% EDTA forced through the sinter in reverse flow will usually clear them effectively. Over-use of alkaline cleaning solution should be avoided, as this tends to erode the glass.



## NEUTRAL DETERGENT FIBRE (CELL WALLS)

### *Analysis of results*

#### Data recording

**Table 3.7 Example of data record sheet**

Sample	Sample weight (g)	Dry matter content (%)	Crucible weight (g)	Crucible + fibre weight (g)	Neutral detergent fibre DWB* (%)
JS1	1.0345	85.04	10.0000	10.0436	4.96

**Note:** \*the filter paper weight may be substituted for the crucible weight.

#### Calculation

$$\% \text{ neutral detergent fibre (as received)} = \frac{\text{weight of crucible* and fibre} - \text{weight of crucible*} \times 100}{\text{weight of sample}}$$

In order to adjust to a dry basis:

$$\% \text{ neutral detergent fibre (dry weight basis)} = \frac{\% \text{ cell walls (as received)} \times 100}{\% \text{ dry matter}}$$

### *International standards*

None known for neutral detergent fibre.

Codex standard for gari (CODEX STAN 151–1985) states that the crude fibre content shall not exceed 2% w/w.

### *References*

AMERICAN ASSOCIATION OF CEREAL CHEMISTS (1983) *Approved Methods of the AACC*. St Paul, Minnesota, USA: American Association of Cereal Chemists.

VAN SOEST, P. J. (1963) Use of detergents in the analysis of fibrous feeds. II. A rapid method for the determination of fibre and lignin. *Journal of Official Agriculture Chemists*, **46**(5): 829.

## **Oils and fats**

### *Introduction*

#### Rationale

Although oils and fats do not form a large component of fresh NGSS, they may be introduced during the processing procedure. For example, gari is often roasted with palm oil; biscuits can contain fat.

#### Suitability

The method described is suitable for processed products which have been fried in oils and fats. It is designed for dry samples, although wet samples can be analysed after treatment with anhydrous sodium sulphate.

### *Limitations*

The fat content of foods consists of free lipid constituents which can be extracted by less polar solvents such as light petroleum fractions and diethyl ether, and bound lipid components which require more polar solvents such as alcohols. Therefore, the amount of lipid found will depend on the method of analysis used.

The method is not suitable for samples containing milk powder.

### Principles

The free lipid content (neutral fats-triglycerides) of samples and free fatty acids can be determined by extracting the dried material with a light petroleum fraction in a continuous extraction apparatus. The solvent is distilled off and the extract is dried and weighed.

## Requirements

### Equipment

- Analytical balance accurate to four decimal places
- Laboratory oven which can be set at 70 °C and 103 °C
- Laboratory balance accurate to two decimal places
- Quickfit 100 ml Soxhlet extractor EX5/63
- Quickfit condenser, Allihn with 40/38 cone CX7/06/SC
- Quickfit 250 ml round-bottomed short-neck flask with 24/29 socket
- Heating mantle or water-bath
- Solvent distillation apparatus

### Consumables

- Extraction thimbles, cellulose 33 x 100 mm
- Cotton/glass wool
- Anti-bumping granules
- Petroleum spirit

### Hazardous chemicals

*Petroleum ether: harmful if inhaled or swallowed; vapour is irritating to eyes, skin and upper respiratory tract; avoid inhalation and contact with eyes and skin; extremely flammable; keep away from heat, sparks and open flame.*

## Procedure

### Determination of free oil and fats

Dry 33 x 100 mm extraction thimble in an oven at 70 °C overnight.

Weigh accurately to nearest 0.1 g, approximately 10 g of sample into extraction thimble. If the sample is semi-solid or wet, add anhydrous sodium sulphate until it becomes powdery and dry before transferring it to the extraction thimble.

Place a small ball of cotton wool/glass wool into top of thimble to prevent loss of the sample. Insert the thimble into a Quickfit Soxhlet extractor. Add anti-bumping granules to a 250 ml round-bottomed flask and weigh accurately. Add 120 ml of petroleum spirit to flask and assemble apparatus. Connect Quickfit condenser to the Soxhlet extractor and reflux for 3 h on the heating mantle.

Evaporate off solvent (it can also be distilled off). Heat the flask and oil/fat for 30 min in oven at 103 °C.

Cool flask and contents to room temperature in a desiccator. Weigh accurately and determine weight of oil/fat collected.

## Analysis of results

### Data recording

**Table 3.8 Example of data record table for oil/fat content**

Sample	Dry matter (%)	Sample weight (g)	Weight of flask (g)	Weight of flask + oil/fat (g)	Weight of extract (g)	Oil/fat dry weight basis (%)
RM1	85.67	10.23	14.0030	15.0456	1.0426	11.90

### Calculation of free oils and fats

$$\% \text{ oil/fat wet weight basis} = \frac{\text{weight extract (g)}}{\text{sample weight (g)}} \times 100$$

$$\% \text{ oil/fat dry weight basis} = \frac{\% \text{ fat, wet weight basis}}{\% \text{ dry matter}} \times 100$$

### *International standards*

None known.

### *References*

HELRICK, K. (ed.) (1990) *Official Methods of Analysis*. 5th edn. Association of Official Analytical Chemists, 1298.

## **Starch, reducing and non-reducing sugars – titration method**

### *Introduction*

#### Rationale

Starch is a major component of NGSS. The quality of the fresh root is greatly influenced by the quality and quantity of starch present. The following method allows the determination of starch, reducing and non-reducing sugars using a titration procedure. An alternative colorimetric method is described in *Starch, reducing and non-reducing sugars – spectrophotometric method* below.

#### Suitability

The methods described permit the accurate determination of reducing and non-reducing sugars and starch in fresh and processed NGSS.

#### Limitations

This method is designed to be used with sample solutions containing between 1 and 60 mg of sugar (as reducing sugar). Aqueous extracts of samples need to be prepared before analysis. To detect low levels of sugars, a sample concentration step may need to be employed. Starch can be determined after hydrolysis to reducing sugars.

#### Principles

Sugars possessing in their structure free aldehyde or ketone groups react as weak reducing agents and are termed reducing sugars. All monosaccharides, and the disaccharides maltose, lactose and cellobiose, are reducing sugars. Disaccharides such as sucrose, and raffinose and oligosaccharides, are composed of simple sugars linked together through their aldehyde and ketone groups and are therefore considered as non-reducing sugars.

Sugars can be estimated by their ability to reduce Cu (II) to Cu (I) in alkaline solution. The Luff-Schoorl method uses an alkaline reagent containing cupric citrate. After boiling with a solution containing reducing sugars, potassium iodide and acid are added after cooling and the liberated iodine, which is equivalent to the unreduced copper, is titrated with thiosulphate.

One of the advantages of this method is that glucose and fructose show exactly the same reducing properties.

### *Requirements*

#### Equipment

- Hot-plate
- Quickfit Erlenmeyer flask, 250 ml with a 24/29 socket (FE250/3)
- Quickfit condenser, double surface (Davies) with 24/29 cone (CX 5/23SC)
- Cooling tray with water running through it; depth of water such that flasks just avoid floating
- Burette, 25 ml and stand
- Pipettes, 25 ml, Grade A bulb
- Pipette, 25 ml, graduated
- Dropping pipette calibrated at 1 ml
- Top-pan laboratory balance accurate to two decimal places

## Consumables

- Boric acid
- AR Hydrochloric acid,
- Lintners soluble starch
- Sodium carbonate, anhydrous
- Citric acid
- Copper sulphate
- Potassium iodide
- Anti-bumping granules
- Thiosulphate solution 0.1 n (standard volumetric solution)
- Zinc acetate
- Acetic acid (glacial)
- Potassium ferrocyanide trihydrate
- Glass fibre filter papers
- Benzoic acid
- Glucose

## Hazardous chemicals

<i>Acetic acid:</i>	<i>harmful if swallowed, inhaled, or absorbed through skin; destructive to human tissue, upper respiratory tract, eyes and skin; use in a fume hood; avoid contact with skin and eyes; avoid inhalation of vapour; corrosive; keep away from heat and open flame.</i>
<i>Benzoic acid:</i>	<i>may be harmful if swallowed, inhaled, or absorbed through skin; causes irritation of the upper respiratory tract, eyes and skin; avoid contact with skin and eyes; avoid inhalation of dust.</i>
<i>Boric acid:</i>	<i>may be harmful if swallowed, inhaled, or absorbed through skin; readily absorbed through skin; avoid contact with skin and eyes, and inhalation.</i>
<i>Citric acid:</i>	<i>may be harmful if swallowed, inhaled, or absorbed through skin; causes irritation of the upper respiratory tract, eyes and skin; avoid contact with skin and eyes; avoid inhalation of dust.</i>
<i>Copper sulphate:</i>	<i>harmful if swallowed or inhaled; causes severe irritation of the eyes; irritating to upper respiratory tract; use in a fume hood; avoid contact with skin and eyes and inhalation of dust; toxic.</i>
<i>Hydrochloric acid:</i>	<i>may be fatal if swallowed, inhaled, or absorbed through skin; destructive to human tissue, upper respiratory tract, eyes and skin; avoid contact with skin and eyes and inhalation of vapour; corrosive; toxic.</i>
<i>Potassium ferrocyanide trihydrate:</i>	<i>may be harmful if swallowed, inhaled, or absorbed through skin; may cause irritation; avoid contact with skin and eyes and inhalation of dust.</i>
<i>Potassium iodide:</i>	<i>harmful if swallowed, inhaled, or absorbed through skin; irritating to upper respiratory tract, eyes and skin; use in a fume hood; avoid contact with skin and eyes; avoid inhalation of dust; possible teratogen.</i>
<i>Sodium carbonate, anhydrous:</i>	<i>may be harmful if swallowed, inhaled, or absorbed through skin; irritating to upper respiratory tract, eyes and skin; avoid contact with skin and eyes and inhalation of dust.</i>
<i>Thiosulphate solution:</i>	<i>may be harmful if swallowed, inhaled, or absorbed through skin; irritating to eyes and skin; avoid contact with skin and eyes, and inhalation of dust; avoid contact with acid.</i>
<i>Zinc acetate:</i>	<i>may be harmful if swallowed, inhaled, or absorbed through skin; irritating to eyes and possibly skin; avoid contact with skin and eyes.</i>

## Procedure

### Preparation of reagents

**Copper reagent.** Dissolve 144.0 g of anhydrous sodium carbonate in 400 ml of hot distilled water in a 1 l conical flask. Swirl the flask a few times and stir until the sodium carbonate has completely dissolved. Dissolve 50.0 g of citric acid in 200 ml of distilled water. Also dissolve 25.0 g of copper sulphate in 200 ml of distilled water. Add the citric acid and copper sulphate solution to the sodium carbonate solution slowly, continuously agitating. Cool with tap water and make up to 1 l.

**Approximately 6 N hydrochloric acid.** Dilute concentrated Analar hydrochloric acid with an equal volume of distilled water.

**0.1 N thiosulphate solution.** Make up from a concentrated volumetric solution. This is only stable for a week.

**Starch indicator, 2% in boric acid.** Tip 7.5 g boric acid into a 500 ml conical flask. Measure 250 ml water into a cylinder and add about 200 ml to the flask. Weigh 5.0 g of Lintners soluble starch into a 100 ml beaker, and make a smooth lump-free suspension by stirring it with a little of the water. Boil the boric acid solution, remove it from the flame, and let it simmer on the hot-plate. Add the starch slowly and wash in with the rest of the water. Simmer for about 5 min then cool. The starch solution should be perfectly clear; it keeps for a considerable time.

**Carrez solution I.** Dissolve 21.9 g zinc acetate dihydrate in water containing 3 g acetic acid; make up to 100 ml with water.

**Carrez solution II.** Dissolve 10.6 g potassium ferrocyanide trihydrate in water; make up to 100 ml with water.

**Apparatus.** Mount a 250 ml 24/26 conical flask containing 50 ml of water together with a 24/26 joint reflux condenser on a retort stand and set the hot-plate to allow gentle boiling. Prepare a cooling tray, with condenser water running through it, the depth of water such that the flask does not float.

### Sample preparation and clarification

Grind the sample to a fine powder or mince to a slurry. For analysis of reducing and non-reducing sugars, transfer a weighed sample to a wide-necked 200 ml volumetric flask (the amount required will depend on its sugar content). Add about 150 ml of hot water and keep warm with shaking to extract the water-soluble matter. Clarify the solution by adding 5 ml Carrez I solution followed by 5 ml Carrez II solution. Make up to the mark, mix and filter through a glass fibre filter paper. If necessary, dilute the filtrate such that a portion contains 15–60 mg reducing/non-reducing sugar.

For analysis of starch, add ground or minced sample directly to 250 ml Erlenmeyer flask, and hydrolyse as given below.

### Luff-Schoorl method for sugar determination

**Determination of reducing sugars** Measure out 25 ml of copper reagent into each of two 250 ml Quickfit Erlenmeyer flasks. To one (the blank) add 25 ml of water. To the other, add clarified sample solution (0–60 mg glucose/fructose) and enough water to make up to 25 ml.

Add a few anti-bumping granules and transfer to the hot-plate; lower the condenser, then boil gently for 10 min. Transfer with tongs to the cooling bath for 5 min. Add the 3 g of potassium iodide into the cooled flask, swirl to dissolve, and with constant swirling add, from a measuring cylinder, 20 ml of 6 N hydrochloric acid.

Titrate contents of flask with 0.1 N thiosulphate until the iodine colour nearly disappears. Add 1 ml of starch indicator and titrate until the blue colour changes to give a white or faint yellow precipitate of cuprous iodide with no trace of blue.

Titrate the blank (which should be less than 25 ml); subtract sample titration = T ml of thiosulphate.

**Determination of non-reducing and reducing sugars** Non-reducing sugars, such as sucrose, can be completely hydrolysed to reducing sugars by refluxing in dilute acid. Measure out the clarified sample (the amount is dependent on the concentration) into a 250 ml Quickfit Erlenmeyer flask. Add 2 ml of 1 N hydrochloric acid and make up to 20 ml using distilled water; reflux on the hot-plate for 10 min. Cool and add 2 ml of 1 N sodium hydroxide and 3 ml water. Add the copper reagent and continue as for reducing sugars.

**Determination of total sugars, including starch** Measure out the sample into a 250 ml flask, add 20 ml of 1 N hydrochloric acid and reflux on the hot-plate for 2 h. Cool and add 20 ml of 1 N sodium hydroxide. Add the copper reagent and continue as for reducing sugars.

### Standards

Prepare a standard glucose solution containing 5 mg/ml using the following procedure. Dissolve 1 g of benzoic acid (preservative) in 500 ml of water by warming, then allow the (saturated) solution to cool. Weigh out 2.5 g of glucose

(preferably dried in a desiccator) into a 50 ml beaker. Dissolve in a little hot benzoic acid, transfer to a 500 ml flask and cool. Make up to volume with benzoic acid. This solution keeps indefinitely in a sealed container.

### Analysis of results

#### Calculation of reducing sugars

Calculate reducing sugars directly from Table 3.9 which relates titration (T) and sugar content (G). It will be seen that T is not proportional to G. In fact the ratio T/G decreases with increase in T or G. To use the table, select the appropriate value of the ratio T/G corresponding to the titration T and divide T by this value.

It will be seen that an error of 1% is possible, which is very satisfactory.

For example, say the initial sample size was 20 g and it was initially extracted in 200 ml water. 25 ml of this extract was analysed for reducing sugars by the Luff-Schoorl method. A titration of 10 ml was obtained. From Table 3.9, the amount of reducing sugar in 25 ml of extract is 25 mg, i.e., each ml of extract contains 1 mg of reducing sugar.

$$\begin{aligned} \text{Amount of reducing sugar in} \\ \text{original sample} &= \frac{\text{Reducing sugar (mg) from Table 3.9}}{\text{Weight of sample (g)}} \times \frac{\text{Total volume of extract (ml)}}{\text{Volume of extract analysed (ml)}} \\ &= \frac{25}{20} \times \frac{200}{25} = 10 \text{ mg/g} \end{aligned}$$

#### Calculation of non-reducing sugars

Non-reducing sugars are calculated by subtracting reducing sugar content from the non-reducing/reducing sugar content.

#### Calculation of starch content

Starch content is calculated by subtracting the reducing sugar and non-reducing sugar contents from the total sugar content.

**Table 3.9 Converting titration volume to sugar content**

Titration ml of 0.1 N thiosulphate (T)	mg glucose or fructose (G)	Ratio (T/G)
1	2.4	0.417
2	4.8	0.417
3	7.2	0.417
4	9.7	0.412
5	12.2	0.410
6	14.7	0.408
7	17.2	0.407
8	19.8	0.404
9	22.4	0.402
10	25.0	0.400
11	27.6	0.399
12	30.3	0.396
13	33.0	0.394
14	35.7	0.392
15	38.5	0.390
16	41.3	0.387
17	44.2	0.385
18	47.1	0.382
19	50.0	0.380
20	53.0	0.377
21	56.0	0.375
22	59.1	0.372
23	62.2	0.370

### Examples

The following results were obtained with a standard solution of glucose.

**Table 3.10 Results table and data handling for sugar determination**

Glucose added (mg)	Titration T (ml)	Factor from Table 3.9	Calculated glucose (mg)	Percentage error (%)
9.01	3.68	0.417	8.8	- 2.2
18.02	7.36	0.406	18.1	+ 0.5
27.02	10.84	0.399	27.2	+ 0.7
36.03	14.16	0.392	36.1	+ 0.3
45.04	17.17	0.384	44.7	- 0.7
54.05	20.27	0.377	53.8	- 0.5

To determine the accuracy of the starch estimation method, the following experiment was carried out:

60 ml of 2% w/v soluble starch was refluxed on a hot-plate with 200 ml of 1 N hydrochloric acid. 10 ml samples were taken at set times and rapidly cooled in ice. Samples were transferred to 250 ml flasks and 7.7 ml of 1 N sodium hydroxide, 7.3 ml of water and copper reagent were added. Reducing sugar estimations were carried out. Samples were taken after 0.0, 0.5, 1.0, 1.5, 2.0, 3.0 and 3.5 h of refluxing. The dry weight of Lintner's starch was also measured and found to be 86% of total weight.

**Table 3.11 Example data handling for reducing sugars and starch determinations**

Hours refluxing	Titration (ml)	Reducing sugar (mg/ml)	Total starch refluxed (g)	Percentage of theoretical* yield (1.333 g)
0.0	24.5	0.60	0.016	1.20
0.5	9.6	38.92	1.012	75.92
1.0	8.1	43.19	1.123	84.25
1.5	8.0	43.48	1.130	84.77
2.0	7.8	44.06	1.146	85.97
3.0	7.9	43.62	1.134	85.07
3.5	8.0	43.48	1.130	84.77
Blank				24.75

**Note:** \*1.2 g of starch will give approximately the equivalent reducing power of 1.333 g of glucose.

As the Lintner's starch was found to have a dry weight of 86%, the theoretical value of 85.97% after refluxing for 2 h is a very satisfactory result.

### International standards

None known.

### References

EGAN, H., KIRK, R. and SAWYER, R. (eds) (1981). The Luff-Schoorl method. Sugars and preserves. pp. 152–153. In: *Person's Chemical Analysis of Foods*. 8th edn. Harlow, UK: Longman Scientific and Technical.

This method has been adapted from that described in:

BROWNE, C.A. and ZERBON, F. W. (1941) *Physical and Chemical Methods of Sugar Analysis*. 3rd edn. New York: John Wiley and Sons.

The Luff-Schoorl method is approved by the European Community (Directive 79/786/EEC) for testing sugar solutions.

## Starch, reducing and non-reducing sugars – spectrophotometric method

### Introduction

#### Rationale

Starch is a major component of NGSS. The quality of the fresh root is greatly influenced by the quality and quantity of starch present. The following method allows the determination of starch, reducing and non-reducing sugars using a colorimetric method. An alternative method is described in *Starch, reducing and non-reducing sugars—titration method*, above.

### Suitability

This method allows the accurate determination of reducing and non-reducing sugars and starch in fresh and processed NGSS.

### Limitations

This assay is designed to be used with sample solutions containing between 0 and 25 mg of sugars (as reducing sugars). To detect high levels of sugar, a sample dilution step may be employed. Starch can be determined after acid hydrolysis to reducing sugars.

This method is only suitable for a well-appointed laboratory with a spectrophotometer.

### Principles

Under alkaline conditions, reducing sugars reduce ferric cyanide to ferrocyanide, which reacts with ferric ions to form a yellow colour. This reaction is then utilized in a colorimetric assay.

### Requirements

#### Equipment

- Amber dispenser, 8 ml
- Analytical balance
- Automatic dispensers, 8 ml; 5 ml; 2.5 ml (optional)
- Automatic pipettes, 5 ml; 10 ml
- Beakers, 100 ml; 250 ml; 500 ml
- Boiling water-bath
- Conical flask, 100 ml
- Desiccator
- Filter funnels
- Flat-bottomed flask
- Flat-bottomed flask, 500 ml
- Glass pipette, 25 ml
- Volumetric flasks, Grade A, 10 ml; 50 ml; 200 ml; 250 ml; 1000 ml
- Glass stoppered tubes, 50 ml
- Metal test-tube rack
- Oven measuring up to 60 °C
- pH meter
- Quartz cuvette
- Soxhlet extractor
- Tongs
- UV/VIS spectrophotometer
- Test-tube rack for 50 ml tubes
- Freeze drier (optional)

#### Consumables

- Amyloglucosidase EC 3.2.1.3. from *Rhizopus* spp., Sigma
- Sodium acetate, anhydrous
- Sodium carbonate, anhydrous



## STARCH, REDUCING AND NON-REDUCING SUGARS – SPECTROPHOTOMETRIC METHOD

- Cellulose extraction thimbles
- Ethanol
- Glacial acetic acid
- Glass balls, 3 mm diameter
- Glucose
- Hydrochloric acid
- Non-absorbent cotton wool
- pH standards
- Potassium ferricyanide (potassium hexacyanoferrate(III))
- Glucose standard solution 1000 mg/l, Sigma
- Sodium hydroxide
- Watch glass
- Whatman GF/A filter paper
- Liquid nitrogen (optional)

### Hazardous chemicals

<i>Acetic acid:</i>	<i>harmful if swallowed, inhaled, or absorbed through skin; destructive to human tissue, upper respiratory tract, eyes and skin; use in a fume hood; avoid contact with skin and eyes; avoid inhalation of vapour; corrosive; keep away from heat and open flame.</i>
<i>Ethanol:</i>	<i>harmful if swallowed, inhaled, or absorbed through skin; causes irritation; vapour is irritating to eyes and upper respiratory tract; flammable; keep away from heat and open flame.</i>
<i>Hydrochloric acid:</i>	<i>may be fatal if swallowed, inhaled, or absorbed through skin; destructive to human tissue, upper respiratory tract, eyes and skin; avoid contact with skin, and eyes, and inhalation of vapour; corrosive; toxic.</i>
<i>Potassium ferricyanide:</i>	<i>may be harmful if swallowed, inhaled, or absorbed through skin; may cause irritation; avoid contact with skin and eyes; avoid inhalation of dust.</i>
<i>Sodium acetate:</i>	<i>may be harmful if swallowed, inhaled, or absorbed through skin; causes irritation of the upper respiratory tract, eyes and skin; avoid contact with skin and eyes; avoid inhalation of dust.</i>
<i>Sodium carbonate, anhydrous:</i>	<i>may be harmful if swallowed, inhaled, or absorbed through skin; irritating to upper respiratory tract, eyes and skin; avoid contact with skin and eyes; avoid inhalation of dust.</i>
<i>Sodium hydroxide:</i>	<i>harmful if swallowed, inhaled, or absorbed through skin; destructive to human tissue, upper respiratory tract, eyes and skin; use in a fume hood; avoid contact with skin and eyes; avoid inhalation of vapour.</i>

### Procedure

Determine the moisture content prior to this analysis.

#### Extraction of soluble sugars

Label the cellulose extraction thimbles with pencil, and weigh using a tared beaker. Weigh 1 g of freeze-dried powder or flour sample into the thimble, and plug the sample with non-absorbent cotton wool. Record the weight of the sample + thimble + cotton wool.

Transfer 200 ml of 85% v/v ethanol into a flat-bottomed flask, with three glass balls (3 mm diameter). Label the flask with a number corresponding to the thimbles. Place the weighed thimble into a Soxhlet extractor and reflux for 1.5–2 h.

Allow the apparatus to cool, and remove the extraction thimble, using tongs. Place the thimble in a warm oven (at about 60 °C) to dry to constant weight. This material is insoluble in ethanol, and should be stored in a desiccator for subsequent determination of starch (*see Starch content*, below).

The liquid left in the extraction equipment is a solution of ethanol-soluble material. Distil off a small volume (one extractor-full) of ethanol, allow to cool and discard (or recycle). Add an equal volume of distilled water to the solution in the flask. Re-assemble the apparatus and continue to distil off the ethanol, discarding after cooling. Continue to distil off and discard the ethanol until water is seen on the sides of the condenser.

The solution is now aqueous and should be made up to a known volume by washing carefully into a 250 ml Grade A volumetric flask, labelled accordingly. Be careful not to transfer the glass balls to the volumetric flask. Solutions can be stored in the refrigerator if necessary for up to 48 h, or the determination of reducing and total sugars can proceed at once. Immediately before analysis, ensure that the solution is at room temperature, and mix well.

*NOTE:* At this stage, the hydrolysis for the total sugars should be carried out (*see below*).

### Reducing sugars

**Preparation of reagents** Solution A: weigh 12.5 g potassium ferricyanide (potassium hexacyanoferrate(III)) and 10.0 g anhydrous sodium carbonate into a 100 ml beaker, add distilled water to dissolve, pour into a 250 ml labelled, Grade A volumetric flask and make up to volume with distilled water.

Solution B: weigh 87.5 g anhydrous sodium carbonate into a 500 ml beaker, add distilled water to dissolve, pour into 1000 ml labelled, Grade A volumetric flask and make up to volume with distilled water.

Ferricyanide reagent: (make this up weekly): pipette 25 ml of Solution A and 100 ml Solution B into a 1000 ml labelled, Grade A volumetric flask, and make up to volume with distilled water. Place in amber automatic dispenser (of at least 8 ml capacity).

**Calibration curve** Weigh accurately 1 g of glucose (dried to constant weight) into a 100 ml beaker and dissolve by adding distilled water. Pour carefully into a 200 ml labelled, Grade A volumetric flask, rinse beaker several times into the flask to transfer all glucose and make up with distilled water. This standard solution contains 5 mg glucose/ml.

To prepare a range of standard solutions, label 50 ml Grade A volumetric flasks and add the following volumes of the 5 mg/ml glucose solution using an automatic pipette (e.g. Finnpiquette): 1.0 ml, 2.5 ml, 4.0 ml, 5.0 ml. Make up to volume with distilled water.

Add 2 ml (use automatic pipette) of each standard solution to 8 ml of ferricyanide reagent in, 50 ml Quickfit glass-stoppered boiling tubes, in duplicate. This produces solutions containing 200 µg, 500 µg, 800 µg and 1000 µg of glucose in the 10 ml reaction mixture. Also prepare blank controls in duplicate containing 2 ml distilled water and 8 ml ferricyanide reagent. Mix the contents of all tubes. Boil for 15 min in a boiling water-bath, using a metal test-tube rack (ensure that stoppers fit loosely). Cool rapidly by placing in a bath of cold water, and mix well.

Measure the absorbance of the mixture at 380 nm with distilled water as the reference/zero, using a quartz cuvette. A graph of weight of glucose in the reaction mixture against absorbance can then be plotted.

**Glucose standard check** As a check, take 1 ml of Sigma standard glucose solution (1000 mg/l) in saturated benzoic acid, and place this in a labelled 10 ml Grade A volumetric flask. Make up to volume using distilled water and mix. Mix 2 ml of this (1 mg/10 ml) solution with 8 ml of the ferricyanide reagent in a 50 ml Quickfit, stoppered boiling tube and mix. Carry this out in duplicate and continue as above. This absorbance is for a solution containing 200 µg glucose in the 10 ml reaction mixture.

**Analysis of aqueous extracts** Mix 2 ml of the aqueous extract (prepared as described above) with 8 ml of the ferricyanide reagent in a labelled, 50 ml, Quickfit, stoppered boiling tube. Carry this out in duplicate for each aqueous extract. For each sample, prepare a blank, using 2 ml of sample and 8 ml of water. Mix contents of all tubes. Boil for 15 min in a boiling water-bath, using a metal test-tube rack. Cool rapidly by placing in a bath of cold water, and mix well.

Measure the absorbance of the samples and sample blanks at 380 nm, with distilled water as the reference/zero and using a quartz cuvette. Read both the sample blank and the sample, and deduct the reading for the blank from that for the sample.

The absorbance value obtained when read off the calibration plot will give a value for the amount of reducing sugar present in the reaction mixture, which, after calculation, will give a value for the amount of sugar in the original tissue. *See Analysis of results* below for calculation.

### Total sugars

The total sugar content of the original sample can be determined by hydrolysing an aliquot of the aqueous extract prepared (*see Extraction of soluble sugars* above), and then using the ferricyanide method to determine the sugars.

To hydrolyse the aqueous extract, take 25 ml of the extract (in a glass pipette) and place in a labelled, 100 ml beaker. Add 5 ml concentrated hydrochloric acid (using an automatic pipette) to the beaker, and cover with a watchglass. Leave for 24 h at

room temperature. Neutralize the solution to pH 7.0 by adding 5 M NaOH. Pour into a labelled 50 ml grade A volumetric flask, rinse the beaker into the flask using distilled water, and make up to volume using distilled water.

### Starch content

The starch content of the alcohol-insoluble material obtained using the procedure described in *Extraction of soluble sugars* above can be determined using the ferricyanide method, and the appropriate calibration curve. First the starch must be hydrolysed, which can be carried out using either acid or enzyme.

**Acid hydrolysis** Prepare a solution of 0.7 M hydrochloric acid by pipetting 150 ml of 35% hydrochloric acid into a 2000 ml labelled, Grade A volumetric flask. Make up to volume using distilled water. Prepare a solution of 5 M NaOH. Weigh 50 g of sodium hydroxide pellets into a 250 ml conical flask. Dissolve by adding distilled water and pour into a 250 ml labelled, Grade A volumetric flask. Make up to volume.

Weigh 200 mg of the dry alcohol-insoluble material (from the thimble) into dry flat-bottomed flasks, labelling each flask. To each flask add 110 ml of 0.7 M HCl and three glass balls. Place over a boiling water-bath, in a fume cupboard if possible. Boil for 2.5 h, shaking the flasks periodically to ensure proper mixing of the sample and acid. Ensure that the level of the liquid is maintained by adding distilled water to the flask. After refluxing, allow the flasks to cool. Neutralize to pH 7.0 by adding 5 M NaOH solution, using a pH meter. Rinse the electrode back into the solution following the pH adjustment.

Label 500 ml Grade A volumetric flasks. Pour the hydrolysate into the flasks and rinse each flat-bottomed flask into the volumetric flasks using distilled water. Make up to volume using distilled water. Ensure that the solution is well mixed.

Filter a 50 ml aliquot of the hydrolysate through glass microfibre filter paper (Whatman GF/A) into a 100 ml labelled conical flask. The starch in the original sample can now be determined as reducing sugar, using the ferricyanide method. Carry out sugar analysis within 48 h of hydrolysis.

**Enzyme hydrolysis** Prepare a 10 mg/ml solution of amyloglucosidase (Amyloglucosidase EC3.2.1.3 from *Rhizopus* spp. obtained from Sigma Co., No. A 7255). Weigh 1 g of the enzyme into a 50 ml beaker. Dissolve by adding distilled water, and pour into a 100 ml, labelled, Grade A volumetric flask. Make up to volume with distilled water and mix well. Filter this solution through glass microfibre filter paper (Whatman GF/A). Keep in a refrigerator for up to 1 week. Place in an automatic dispenser (5 ml).

Prepare a 4 M acetate buffer, pH 4.8. Weigh 164 g anhydrous sodium acetate into a 500 ml beaker. In a fume cupboard, dissolve the sodium acetate by adding 120 ml glacial acetic acid. Pour into a 1000 ml, labelled Grade A volumetric flask and make up to volume using distilled water. Mix well, adjust to pH 4.8, and place in an automatic dispenser (2.5 ml).

Label a dry, flat-bottomed flask. Weigh 200 mg of the dry alcohol-insoluble material into the flask. An enzyme blank should also be prepared. Add 2.5 ml of 4 M acetate buffer (pH 4.8), 42 ml of distilled water and three glass beads (3 mm) to the flask. Cover the flask with aluminium foil, and place in a boiling water-bath for 1 h, shaking the flask several times during this period.

After boiling, allow to cool to below 50 °C. Add 5 ml (automatic dispenser) of the amyloglucosidase solution. Incubate at 55 °C for 1 h, in a continuous-shaking water-bath. After incubation, pour the hydrolysate into a labelled 500 ml Grade A volumetric flask, rinsing the flask into the volumetric flask using distilled water. Make up to volume using distilled water and mix thoroughly.

Filter a 25 ml aliquot of the hydrolysate through glass microfibre filter paper (Whatman GF/A) into a 100 ml labelled conical flask. The starch in the original sample can now be determined as reducing sugar, using the ferricyanide method. Carry out sugar analysis within 48 h of extraction.

### *Analysis of results*

#### Note on conversion factor from glucose to starch

As the starch values are obtained by reading from a reducing-sugar calibration curve, the values obtained will be for glucose. To convert these values to starch they should be multiplied by 0.9. This is because as the starch is hydrolysed, water is added to each sub-unit, and the ratio of weights of glucose:water is 9:1. Therefore, the starch value will be nine tenths (0.9) of the corresponding value for glucose.

## Data recording

**Table 3.12 Example of data record sheet for reducing sugars, total sugars and starch**

Sample	D1 Dry weight (g)	C1 ( $\mu\text{g}/10\text{ ml}$ )	RS Reducing sugars ( $\text{mg}/\text{g DW}$ )	C2 ( $\mu\text{g}/10\text{ ml}$ )	TS Total sugars ( $\text{mg}/\text{g DW}$ )	C3 ( $\mu\text{g}/10\text{ ml}$ )	A Weight of insoluble material (g)	S Starch in original sample ( $\text{mg}/\text{g}$ )
RL1	1.0050	0.19	23.6	0.40	99.5	400	0.9504	425.5

## Calculation reducing sugars

$$D_1 = \frac{W - M \times W}{100}$$

where: W = original sample weight (g);  
 M = percentage moisture content of original sample;  
 D<sub>1</sub> = dry weight of original sample (g).

$$RS = \frac{(C_1 \times 125)}{D_1} \times 1000 \text{ mg/g dry weight}$$

where: C<sub>1</sub> = concentration of reducing sugars in the 10 ml reaction mixture ( $\mu\text{g}/10\text{ ml}$ ) read from the appropriate calibration graph;  
 RS = weight of reducing sugars in the original sample ( $\text{mg}/\text{g dry weight}$ );  
 125 = conversion factor since the soluble sugars from the original sample are dissolved in 250 ml. From this 250 ml, 2 ml are taken, and the absorbance due to reducing sugars measured;  
 1000 = conversion factor to convert final answer from g/g to mg/g. This conversion factor is not required if C is entered as C  $\mu\text{g}$ .

## Calculation of total sugars

$$TS = \frac{(C_2 \times 25 \times 10)}{D_1} \times 1000 \text{ mg/g dry weight}$$

where: C<sub>2</sub> = concentration of reducing sugars in the 10 ml reaction mixture ( $\mu\text{g}/10\text{ ml}$ ) read from the appropriate calibration graph;  
 TS = weight of total sugars in the original sample ( $\text{mg}/\text{g dry weight}$ );  
 25  $\times$  10 = conversion factor since the soluble sugars from the original sample are dissolved in 250 ml. From this, 25 ml are taken, made up to 50 ml; from this 50 ml, 2 ml are taken, and the absorbance due to reducing sugars in the reaction mixture measured;  
 1000 = conversion factor to convert final answer from g/g to mg/g. This conversion factor is not required if C is entered as C  $\mu\text{g}$ .

## Calculation of starch

*Acid or enzymic hydrolysis*

$$S = \frac{0.9 \times C_3 \times 250 \text{ g/g alcohol-insoluble material}}{D_3}$$

$$\text{Starch in original sample} = \frac{A \times S \times 1000 \text{ mg/g}}{D_1}$$

where: S = weight of starch in the alcohol-insoluble solids (g);  
 C<sub>3</sub> = weight of sugars in the 10 ml reaction mixture ( $\mu\text{g}/10\text{ ml}$ ) from the appropriate calibration graph;  
 D<sub>3</sub> = weight of sample of alcohol-insoluble solids used (200 mg);  
 0.9 = conversion factor from glucose to starch (see above);

## STARCH, REDUCING AND NON-REDUCING SUGARS – SPECTROPHOTOMETRIC METHOD

- 250 = conversion factor since the 200 mg of alcohol-insoluble material from the original sample are dissolved in 500 ml; from this 500 ml, 2 ml are taken, and the absorbance due to reducing sugars in the reaction mixture measured;
- A = weight of alcohol-insoluble solids obtained after extraction (g);
- D<sub>1</sub> = dry weight of original sample;
- 1000 = conversion factor to convert final answer from g/g to mg/g. This conversion factor is not required if C is entered as C µg.

### *International standards*

None known.

### *References*

ASSOCIATION OF OFFICIAL ANALYTICAL CHEMISTS (1984) *Official Methods of Analysis*, 14.023. AOAC.

## **Total ash**

### *Introduction*

#### Rationale

Ash consists of the inorganic constituents of NGSS. The analytical method described here provides an estimation of the mineral matter present in NGSS including metals, salts and trace elements. The ash content can provide an estimate of the quality of the product, since high ash levels may indicate contamination.

#### Suitability

This method is applicable to all types of fresh commodities and their products.

#### Limitations

There are no known limitations to this method.

#### Principles

The ash of a foodstuff is the inorganic residue remaining after the organic matter has been burnt away. It should be noted, however, that the ash obtained is not necessarily of the same composition as the mineral content as there may be some loss from volatilization.

### *Requirements*

#### Equipment

- Muffle furnace
- Silica or platinum dish, approximately 7 cm diameter
- Desiccator
- Analytical balance accurate to four decimal places

### *Procedure*

#### Determination of total ash

Accurately weigh approximately 5 g of sample into a silica (or platinum) dish which has previously been ignited and cooled before weighing.

Ignite the dish and contents gently over a low flame, or under an infra-red lamp, until charred, then transfer to a muffle furnace at 500–550 °C and leave until ash is formed (several hours).

Allow the dish and contents to cool in a desiccator. Reweigh the contents and calculate the amount of ash.

*Analysis of results*

## Data recording

**Table 3.13** Example of data record table for ash content

Sample	Dish weight (g)	Sample weight (g)	Dish + ash weight (g)	Weight of ash (g)	Ash content (%)
JA1	10.0000	15.0345	11.6780	1.6780	11.16

## Calculation of total ash content

Calculate and express the results as percentage total ash.

$$\text{Percentage ash content} = \frac{\text{Weight of ash}}{\text{Weight of sample}} \times 100$$

*International standards*

Codex standard for cassava gari (CODEX STAN 151–1985) states that the ash content shall not exceed 2.75% w/w.

Codex standard for cassava flour (CODEX STAN 176–1991) states that the ash content shall not exceed 3% m/m.

*References*

EGAN, H., KIRK, R. and SAWYER, R. (eds) (1981) General chemical methods, ash and mineral matter. pp. 11–12. In: *Person's Chemical Analysis of Foods*. 8th edn. Harlow, UK: Longman Scientific and Technical.

## Section 3.3 Microbiological analyses

### INTRODUCTION

The microbiological profile of NGSS and their products can be determined by a trained operative carrying out selected, internationally recognized tests. The number and type of specific groups of micro-organisms in a product indicates the way it has been handled at all stages, post-harvest, e.g. storage, fermentation, processing, packaging, transportation. The level of spoilage micro-organisms can be used to predict the potential shelf-life of the product.

The presence and numbers of faecal coliform bacteria are used to indicate the occurrence of contamination of the NGSS or their products by faecal material from warm-blooded animals. The presence of this group of bacteria in a processed food is indicative of poor hygienic procedures during handling/production. International legislation requires that food for human consumption is free from pathogenic bacteria, such as *Salmonella*. However, low numbers of the spore-forming pathogens *Clostridium perfringens* and *Bacillus cereus* are ubiquitous in some processed dried products. Their presence is usually acceptable, albeit at low levels of  $10\text{--}10^3$  colony-forming units/g. It is also important that interpretation of microbiological analyses is carried out by experienced personnel.

### AEROBIC PLATE COUNT

#### Introduction

The aerobic plate count provides information on the number of aerobic bacteria present in a sample.

#### *Rationale*

The number and type of bacteria present in a sample can be assessed, subject to appropriate culture medium and incubation temperature. The level of aerobic bacteria will indicate the freshness/potential shelf-life of a product. Types of bacteria in the product indicate how the product has been prepared/handled.

#### *Suitability*

This method is suitable for determining the microbial load of fresh, fermented and processed products. It is important that a representative sample is taken and that a minimum of two, and preferably five, sub-samples are tested from each batch.

#### *Limitations*

This method should be carried out in an authorized laboratory by a trained operative.

#### *Principle*

The sample is mixed together with a sterile diluent, from which a 10-fold dilution series is prepared. Aliquots from a range of dilutions are transferred onto/into an appropriate culture medium and the plates are incubated at a pre-determined temperature for the specific test required, i.e., aerobic thermophilic, aerobic mesophilic, aerobic psychrotrophic or anaerobic mesophilic counts. After the required incubation period, the number of colonies is counted and the level of bacteria present in the sample expressed as colony forming units (cfu)/g.

### Requirements

#### *Equipment*

- Incubator at 30 °C
- Autoclave
- Balance
- Waring blender and base
- Laminar flow cabinet

- Colony counter
- Stomacher and bags (optional)

### *Consumables*

- Petri dishes
- Containers for sterilizing Petri dishes
- Glass bottles with autoclavable lids
- Autoclave tape
- Pipettes, 1.0 ml, blow-out
- Containers for sterilizing pipettes
- Universal containers
- Maximum Recovery Diluent (Oxoid CM733)
- Plate Count Agar (Oxoid CM325)
- Peptone
- Sodium chloride
- Tryptone
- Yeast extract
- Glucose
- Agar
- Alcohol
- Glass beaker
- Glass 'hockey sticks'

### *Hazardous chemicals*

*Ethanol:* may be harmful if inhaled, swallowed or absorbed through skin; irritant; avoid inhalation of vapour and contact with skin and eyes; flammable; avoid heat, open flame or sparks.

*Sodium chloride:* may be harmful if inhaled, ingested or absorbed through skin; irritant to skin, eyes and upper respiratory tract; avoid inhalation of dust and contact with skin and eyes.

Note: Microbiological media may contain potentially hazardous compounds. It is recommended that the manufacturer's instructions and warnings are followed closely.

## **Procedure**

### *Preparation of dilution series*

Macerate 30 g of the sample for 2 min with 270 ml of sterile diluent (Maximum Recovery Diluent – 0.1% peptone + 0.85% sodium chloride, Oxoid) in an Waring blender. If analysing a powder or soft product, use a stomacher. This provides a dilution of  $10^{-1}$ . With a sterile pipette, transfer 1 ml of the  $10^{-1}$  dilution into 9 ml sterile diluent, in a universal container, to give a dilution of  $10^{-2}$ . Repeat this process, diluting the sample to the required level. For example, a fresh product will have a low level of micro-organisms, so the sample should only be diluted to approximately  $10^{-3}/10^{-4}$ . In comparison, a fermented product will contain high levels of bacteria, so will need to be diluted to  $10^{-7}$ .

### *Preparation of Maximum Recovery Diluent*

Formula (Oxoid)

Peptone	1.0 g/l
Sodium chloride	8.5 g/l
Final pH	$7.0 \pm 0.2$

Dissolve the 9.5 g in 1 l of distilled water. Dispense in required volumes (270 ml and 9 ml) and sterilize by autoclaving at 121 °C for 15 min.



## AEROBIC PLATE COUNT

### *The spread plate technique*

Pour plates with 15 ml of molten Plate Count Agar (Oxoid). Allow to set and dry. Pipette 0.1 ml of diluted sample onto the surface of the agar and spread the inoculum evenly with a sterile glass 'hockey stick'. Duplicate plates are prepared for each dilution and, after drying, incubated under appropriate conditions in an inverted position. The specific incubation temperatures include:

- aerobic thermophilic count: 55 °C for 48 h
- aerobic mesophilic count: 30 °C for 72 h
- anaerobic mesophilic count: 30 °C for 72 h
- aerobic psychrotrophic count: 25 °C for 5 days.

### *The pour plate technique*

To duplicate sets of Petri dishes, transfer 1 ml aliquots of the diluted sample. Pour in 15 ml of agar which has been melted and held at 45 °C. Mix well and allow to set on a level surface. Dry the plates and incubate, in an inverted position, according to the specific test requirement.

### *Preparation of Plate Count Agar*

Formula (Oxoid)

Tryptone	5.0 g/l
Yeast Extract	2.5 g/l
Glucose	1.0 g/l
Agar	9.0 g/l
Final pH	7.0 ± 0.2

Suspend the 17.5 g in 1 l of distilled water. Dissolve by bringing to the boil with frequent stirring, mix and distribute into final containers. Sterilize by autoclaving at 121 °C for 15 min.

## **Analysis of results**

### *Enumeration of bacteria*

The number of colonies on Plate Count Agar should be counted after the specific incubation time. Counts should be made from plates supporting 30–300 colonies and the mean of the duplicate plates noted. When the spread plate technique has been followed, the mean should be multiplied by 10 to give the number of cfu/ml dilution. This number is then multiplied by the dilution factor to give the number of cfu/g sample. However, if the pour plate technique has been used, the mean of the two numbers need only be multiplied by the dilution factor. The level of bacteria should be expressed as the number of cfu/g of sample (ICMSF, 1978).

## **International standards**

There are no known international standards for NGSS.

The Codex Alimentarius Commission recommended that the product should be handled in accordance with the appropriate sections of the Recommended International Code of Practice – General Principles of Food Hygiene (CAC/RCP1–1969, Rev 2 – 1985). Recommended microbial limits are not presented.

## **References**

### *Sources*

OXOID MANUAL (1990) 6th edn compiled by BRIDSON, E. Y. Basingstoke, UK: Unipath Ltd.

INTERNATIONAL COMMISSION ON MICROBIOLOGICAL SPECIFICATIONS FOR FOODS (ICMSF) (1978). *Micro-Organisms in Foods*. 1. *Their Significance and Methods of Enumeration*, 2nd edn. Toronto, Canada: University of Toronto Press.

CODEX ALIMENTARIUS COMMISSION (1985) *Recommended International Code of Practice—General Principles of Food Hygiene (CAC/RCP1—1969, Rev 2)*.

### *Further reading*

HARRIGAN, W. F. and MCCANCE, M. E. (1976) *Laboratory Methods in Food and Dairy Microbiology*. London: Academic Press.

INTERNATIONAL COMMISSION ON MICROBIOLOGICAL SPECIFICATIONS FOR FOODS (ICMSF) (1978) *Microbial Ecology of Foods. Volume 2—Food Commodities*. London: Academic Press.

INTERNATIONAL COMMISSION ON MICROBIOLOGICAL SPECIFICATIONS FOR FOODS (1988) *Micro-Organisms in Foods 2: Sampling for Microbiological Analysis: Principles and Specific Applications*. Oxford: Blackwell Scientific Publications.

## COLIFORM BACTERIA

### Introduction

Coliform bacteria can grow in the presence of bile salts and produce acid and gas from lactose when incubated at 35 or 37 °C.

### Rationale

Coliform bacteria are used as indicators of poor hygiene/post-processing contamination. Their presence in a food plant indicates that cleaning protocols are not adequate, that a stage of the process, such as pasteurization or sterilization, has failed, or that contamination of the product has occurred after processing.

### Suitability

The following methods can be used to determine the level of coliform bacteria in a wide range of fresh and processed foods.

### Limitations

Procedures should be carried out in an authorized laboratory by a trained operative. This method cannot be used to detect strains of the enteropathogenic *Escherichia coli* group.

### Principles

Coliform bacteria can be determined by a multiple tube technique to predict the Most Probable Number (MPN) of this group of bacteria in a sample, or by a direct plate count using a selective medium. Tests are incubated at 35 °C and results obtained after 24/48 h.

## Requirements

### Equipment

- Incubator at 35/37 °C
- Autoclave
- Balance
- Colony counter

### Consumables

- Universal containers
- Durham tubes
- Petri dishes
- Containers for sterilizing Petri dishes
- Glass bottles with autoclavable lids
- Autoclave tape
- Pipettes, 1.0 ml, blow-out
- Containers for sterilizing pipettes
- MacConkey Broth (purple) (Oxoid CM5a)
- Violet Red Bile Agar (Oxoid CM107)

## COLIFORM BACTERIA

- Alcohol
- Glass beaker and glass 'hockey sticks'

### *Hazardous chemicals*

*Ethanol:* may be harmful if inhaled, swallowed or absorbed through skin; irritant; avoid inhalation of vapour, and contact with skin or eyes; flammable; avoid heat, open flame or sparks.

*Sodium chloride:* may be harmful if inhaled, ingested or absorbed through skin; irritant to skin, eyes and upper respiratory tract; avoid inhalation of dust, contact with skin or eyes.

Note: Microbiological media may contain potentially hazardous compounds. It is recommended that the manufacturer's instructions and warnings are followed closely.

## Procedure

### *Preparation of MacConkey Broth (purple)*

Formula (Oxoid)

Peptone	20.0 g/l
Lactose	10.0 g/l
Bile salts	5.0 g/l
Sodium chloride	5.0 g/l
Bromocresol purple	0.01 g/l
Final pH	7.4 ± 0.2

To prepare single strength broth, add the 40 g to 1 l of distilled water. Distribute into containers fitted with Durham fermentation tubes. Sterilize by autoclaving at 121 °C for 15 min.

### *Determination of Most Probable Number (MPN) of coliform bacteria*

Prepare food sample as described in section *Preparation of dilution series, Aerobic plate count* above. Dilution blanks remaining from the determination of the plate count may be used. Pipette 1 ml of the decimal dilutions into each of the three separate tubes of Oxoid MacConkey Broth (purple) ( $10^{-1}$ ,  $10^{-2}$  and  $10^{-3}$ ). Incubate tubes at 35–37 °C for 24 and 48 h. After 24 h, record tubes showing gas production. Return tubes not displaying gas production to the incubator for an additional 24 h.

### *Preparation of Violet Red Bile Agar*

Formula (Oxoid)

Yeast extract	3.0 g/l
Peptone	7.0 g/l
Sodium chloride	5.0 g/l
Bile Salts No. 3	1.5 g/l
Lactose	10.0 g/l
Neutral red	0.03 g/l
Crystal violet	0.002 g/l
Agar	12.0 g/l
Final pH	7.4 ± 0.2

Suspend the 35.5 g in 1 l of distilled water. Bring to the boil to dissolve completely. No further sterilization is necessary or desirable. Mix well before pouring.

### *Direct plate count for coliform bacteria*

Prepare food sample as described in *Preparation of dilution series, Aerobic plate count* above. Transfer 1 ml of each dilution of food homogenate into a sterile Petri dish. Add to each Petri dish 10–15 ml of Violet Red Bile Agar (VRBA) cooled to 45 °C. Mix contents of plates thoroughly by tilting and rotating each dish. Allow to solidify on a level surface; then add an additional 3–4 ml VRBA as an overlay, completely covering the surface colony formation. Invert and incubate the plates for 24 h at 35 °C.

## Controls

Positive control: *Escherichia coli*

Negative control: *Staphylococcus aureus*

## Analysis of results

### Most Probable Number table

**Table 3.14 Most Probable Number table**

Number of positive tubes			MPN per g	Confidence limits			
10 <sup>-1</sup>	10 <sup>-2</sup>	10 <sup>-3</sup>		99%		95%	
0	1	0	3	<1	23	<1	17
1	0	0	4	<1	28	1	21
1	0	1	7	1	35	2	27
1	1	0	7	1	36	2	28
1	2	0	11	2	44	4	35
2	0	0	9	1	50	2	38
2	0	1	14	3	62	5	48
2	1	0	15	3	65	5	50
2	1	1	20	5	77	8	61
2	2	0	21	5	80	8	63
3	0	0	23	4	177	7	129
3	0	1	40	10	230	10	180
3	1	0	40	10	290	20	210
3	1	1	70	20	370	20	280
3	2	0	90	20	520	30	390
3	2	1	150	30	660	50	510
3	2	2	210	50	820	80	640
3	3	0	200	<100	1900	100	1400
3	3	1	500	100	3200	200	2400
3	3	2	1100	200	6400	300	4800

Calculated from data in de Man (1975).

### Most Probable Number

After 48 h, record tubes showing gas production. The formation of acid and gas after 24 or 48 h is considered sufficient evidence of the presence of coliforms. Record the number of tubes that were positive for coliform organisms. Refer to the MPN table and note the MPN value. For example, positive results in three tubes of 10<sup>-1</sup> dilution, one tube of 10<sup>-2</sup> dilution and zero tubes of 10<sup>-3</sup> dilution will give a number of 310. This would give an MPN of 40/g in the sample examined.

### Direct plate count

Only dark red colonies measuring 0.5 mm or more on uncrowded plates are considered to be coliform bacteria. If possible, count plates with not more than 150 such colonies. Multiply the number of colonies by the dilution to obtain the number of coliform organisms g of sample.

## International standards

There are no known International Standards for NGSS.

## References

### Source

INTERNATIONAL COMMISSION ON MICROBIOLOGICAL SPECIFICATIONS FOR FOODS (ICMSF), (1978) *Micro-organisms in Foods. 1. Their Significance and Methods of Enumeration*. 2nd edn. Toronto, Canada: University of Toronto Press.

DE MAN (1975) The probability of Most Probable Numbers. *European Journal of Applied Microbiology*, **1**: 67.

## COLIFORM BACTERIA

### Other references

AMERICAN PUBLIC HEALTH ASSOCIATION (1978) *Standard Methods for the Examination of Dairy Products* 14th edn. Washington DC, USA: APHA Inc.

AMERICAN PUBLIC HEALTH ASSOCIATION (1976) *Compendium of Methods for the Microbiological Examination of Foods*. Washington DC, USA: APHA Inc.

## FAECAL COLIFORM BACTERIA

### Introduction

Faecal coliform bacteria are bacteria which, in the presence of bile salts or other equivalent selective agents, can grow and produce acid and gas from lactose when incubated at 44–45.5 °C (Harrigan and McCance, 1976).

### Rationale

The presence of faecal coliform bacteria in a food plant/product is indicative of contamination with faecal material from warm-blooded animals.

### Suitability

This method is suitable for confirming the presence of faecal coliforms in a wide range of fresh and processed products.

### Limitations

This method should be carried out in an authorized laboratory by a trained operative.

### Principles

Positive tubes from the test for coliform bacteria (*Coliform bacteria* above) are sub-cultured into selective/diagnostic enrichment broths and incubated at an elevated temperature (44 °C) for 24/48 h. Cultures showing typical results for faecal coliforms are then confirmed as *Escherichia coli* by a selection of biochemical tests.

## Requirements

### Equipment

- Incubator at 35 °C
- Water-bath at 44 °C ± 0.1 °C
- Autoclave
- Balance

### Consumables

- Universal containers
- Durham tubes
- Petri dishes
- Containers for sterilizing Petri dishes
- Glass bottles with autoclavable lids
- Autoclave tape
- Pipettes, 1.0 ml, blow-out
- Containers for sterilizing pipettes
- Brilliant Green Bile (2%) Broth (Oxoid CM31)
- Peptone Water (Oxoid CM9)

- Simmons Citrate Agar (Oxoid CM155B)
- Methyl Red
- Potassium hydroxide
- $\alpha$ -naphthol
- Paradimethylaminobenzaldehyde
- Isoamyl alcohol
- Hydrochloric acid, concentrated

### *Hazardous chemicals*

<i>Ethanol:</i>	<i>may be harmful if inhaled, swallowed or absorbed through skin; irritant; avoid inhalation of vapour and contact with skin and eyes; flammable; avoid heat, open flame or sparks.</i>
<i>Sodium chloride:</i>	<i>may be harmful if inhaled, ingested or absorbed through skin; irritant to skin, eyes and upper respiratory tract; avoid inhalation of dust and contact with skin and eyes.</i>
<i>Potassium hydroxide:</i>	<i>causes severe burns; avoid contact with eyes and skin.</i>
<i><math>\alpha</math>-naphthol:</i>	<i>may be harmful if inhaled, ingested or absorbed through skin; may cause irritation; avoid contact with skin and inhalation; wash thoroughly after handling.</i>
<i>Paradimethylaminobenzaldehyde:</i>	<i>may be harmful if inhaled, ingested or absorbed through skin; causes irritation to eyes and skin; irritating to mucous membranes and upper respiratory tract; avoid breathing in dust and contact with eyes, skin and clothing; light sensitive; store under nitrogen in a cool dry place.</i>
<i>Isoamylalcohol:</i>	<i>harmful if inhaled, ingested or absorbed through skin; vapour irritating to eyes and upper respiratory tract; use in a fume hood; avoid inhalation of vapour and contact with eyes, skin and clothing; flammable liquid; keep away from heat, sparks and open flame.</i>
<i>Hydrochloric acid:</i>	<i>may be fatal if ingested, inhaled or absorbed through skin; destructive to human tissue, upper respiratory tract, eyes and skin; avoid inhalation of vapour and contact with eyes and skin; corrosive; toxic.</i>

Note: Microbiological media may contain potentially hazardous compounds. It is recommended that the manufacturer's instructions and warnings are followed closely.

## **Procedure**

### *Preparation of Peptone Water*

Formula (Oxoid)	
Peptone	10.0 g/l
Sodium chloride	5.0 g/l
Final pH	7.2 $\pm$ 0.2

Dissolve the 15 g in 1 l of distilled water. Mix well and distribute into final containers. Sterilize by autoclaving at 121 °C for 15 min.

### *Preparation of Brilliant Green Bile (2%) Broth*

Formula (Oxoid)	
Peptone	10.0 g/l
Lactose	10.0 g/l
Ox bile (purified)	20.0 g/l
Brilliant green	0.0133 g/l
Final pH	7.4 $\pm$ 0.2

Add the 40 g to 1 l of distilled water. Mix well, distribute into containers fitted with Durham's tubes and sterilize by autoclaving at 121 °C for 15 min.

## FAECAL COLIFORM BACTERIA

### *Determination of Most Probable Number (MPN)*

Select tubes of MacConkey Broth that are positive for gas production as described in *Determination of MPN of coliform bacteria, Coliform bacteria*. Inoculate a loopful of broth from each positive culture into a tube of Brilliant Green Bile (2%) Broth and a tube of Peptone water. Incubate tubes at  $44 \text{ }^{\circ}\text{C} \pm 0.1 \text{ }^{\circ}\text{C}$ . Read Brilliant Green Bile (2%) Broth after 24 and 48 h of incubation. After 24 h incubation, pipette aseptically a 5 ml portion of each Peptone Water tube to a separate tube and test for the presence of indole.

### *Preparation of Kovac's Indole Reagent*

Dissolve 5 g of paradimethylaminobenzaldehyde in 75 ml of isoamyl (or normal amyl) alcohol and add 25 ml of concentrated hydrochloric acid.

### *Indole test*

Inoculate tubes of Peptone Water from pure cultures. Incubate tubes at  $35 \text{ }^{\circ}\text{C}$  for 24 h. Add 0.2–0.3 ml of Indole Reagent to each tube and shake. Leave tubes to stand for 10 min and observe results. A dark red colour in the amyl acetate layer constitutes a positive reaction. An orange colour indicates the probable presence of skatole and may be reported as a  $\pm$  reaction.

### *Further identification of faecal coliforms*

#### IMViC tests

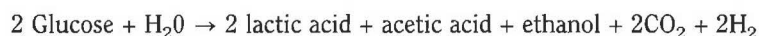
Streak a loopful of each gas-positive broth onto a separate plate of MacConkey Agar. Incubate inverted plates for 24 h at  $35 \text{ }^{\circ}\text{C}$ . Remove a representative colony from each plate and streak cells onto a slope of Nutrient Agar and into a tube of Lactose Broth. Incubate cultures for 24 h at  $35 \text{ }^{\circ}\text{C}$ . Cultures producing gas from lactose should be checked by examining a smear stained by Gram's method to confirm the presence of Gram-negative non-spore forming rods. Use an inoculating needle and the 24 h culture, on Nutrient agar, to inoculate the IMViC media.

#### Indole test

Inoculate tubes of Peptone Water from pure cultures. Incubate tubes at  $35 \text{ }^{\circ}\text{C}$  for 24 h. Continue procedure as described in *Indole test* above.

#### Methyl red test

Inoculate tubes of Buffered Glucose Broth (MRVP) from pure cultures. Incubate tubes at  $35 \text{ }^{\circ}\text{C}$  for 5 days. Pipette 5 ml from each culture to a separate empty container, add 5 drops of methyl red (MR) solution and shake. Record a distinct red colour as MR-positive, a distinct yellow colour as MR-negative and a mixed shade as questionable.



MR-positive organisms produce stable acids maintaining a high concentration of hydrogen ions until a certain concentration is reached and then all activity ceases. MR-negative organisms still produce acids (acetic, lactic and formic), but they have a lower concentration of hydrogen ions because of a reversion towards neutrality due to further degradation of the organic acids to carbonates, and on to carbon dioxide, and possibly to formation of ammonium compounds from the protein present in the medium.

#### Voges-Proskauer test

Inoculate tubes of Buffered Glucose Broth (MRVP) from pure cultures and incubate at  $35 \text{ }^{\circ}\text{C}$  for 48 h. Transfer 1 ml of each culture to a separate empty culture tube and add 0.6 ml of naphthol solution and 0.2 ml of potassium hydroxide solution. Leave for 2 h then note the results; a pink colour indicates a positive test.

#### Citrate test

To determine if an organism is capable of utilizing citrate as the sole source of carbon, inoculate tubes of Simmons Citrate Agar with cells from pure cultures by the stab and streak method. Use a straight needle, since transfer of nutrients can invalidate the test. Incubate tubes at  $35 \text{ }^{\circ}\text{C}$  for 72–96 h. Record visible growth as a positive reaction and no growth as a negative reaction. Growth is usually indicated by a change in the colour of the medium from light green to blue.

*Preparation of MRVP Medium*

## Formula (Oxoid)

Peptone	5.0 g/l
Glucose	5.0 g/l
Phosphate buffer	5.0 g/l
Final pH	7.5 ± 0.2

Add the 15 g to 1 l of distilled water. Mix well, distribute into final containers and sterilize by autoclaving at 121 °C for 15 min.

*Preparation of methyl red solution*

## Formula

Methyl red	0.1 g
Ethanol	300.0 ml
Water	500.0 ml

Dissolve the methyl red in the ethanol and dilute with the water.

*Preparation of potassium hydroxide solution*

## Formula

Potassium hydroxide	40.0 g
Distilled water	100.0 ml

Dissolve the potassium hydroxide in the distilled water.

*Preparation of α-naphthol solution 1%*

## Formula

α-Naphthol	1.0 g
Absolute alcohol	100.0 ml

Dissolve the α-naphthol in the absolute alcohol.

*Preparation of Simmons Citrate Agar*

## Formula (Oxoid)

Magnesium sulphate	0.2 g/l
Ammonium dihydrogen phosphate	0.2 g/l
Sodium ammonium phosphate	0.8 g/l
Sodium citrate, tribasic	2.0 g/l
Sodium chloride	5.0 g/l
Bromothymol blue	0.08 g/l
Agar	15.0 g/l
Final pH	7.0 ± 0.2

Suspend the 23 g in 1 l of distilled water. Bring to the boil to dissolve completely. Sterilize by autoclaving at 121 °C for 15 min. Leave to cool as slants.

*Controls*

Positive control: *Escherichia coli*

Negative control: *Staphylococcus aureus*



*Differentiation of coliforms***Table 3.15** Organisms capable of producing acid and gas from lactose in 48 h at 35 °C\*

	Gas at 44 °C	Indole	MR	VP	Citrate
<i>Escherichia coli</i>					
Type I (typical)	+	+	+	—	—
Type II	—	—	+	—	—
Intermediates					
Type I	—	—	+	—**	+
Type II	—	+	+	—**	+
<i>Enterobacter aerogenes</i>					
Type I	—	—	—	+	+
Type II	—	+	—	+	+
<i>Enterobacter cloacae</i>					
Irregular					
Type I	—	+	+	—	—
Type II	+	—	+	—	—
Type VI	+	—	—	+	+
Irregular other types			Reactions variable		

**Notes:** \* Gas in lactose bile-salt medium at 44–45.5 °C

\*\*Weak positive reactions are occasionally found

**Analysis of results***Determination of Most Probable Number (MPN)*

Cultures producing gas in the presence of bile and indole, at 44 °C, are presumed positive for faecal coliform organisms. Determine the MPN of faecal coliforms/g sample as described in *Determination of MPN* above.

**International standards**

There are no known International Standards for NGSS.

**References***Source*

HARRIGAN, W. F. and McCANCE, M. E. (1976) *Laboratory Methods in Food and Dairy Microbiology*. London: Academic Press.

KOSER, S. A. (1923) Utilisation of salts of organic acids by the colon-aerogenes group. *Journal of Bacteriology*, **8**: 493.

KOVACS, N. (1928) A simplified method for detecting indole formation by bacteria. *Zeitung Immunitatsforsch.*, **56**: 311; *Chemical Abstracts*, **22**: 3425.

LEVINE, M. (1916) On the significance of the Voges-Proskauer reaction. *Journal of Bacteriology*, **1**: 153.

LJUTOV, V. (1961) Technique of methyl red test. *Acta Pathol. Microbiol. Scand.*, **58**: 325.

LJUTOV, V. (1963) Technique of Voges-Proskauer test. *Acta Pathol. Microbiol. Scand.*, **58**: 325.

SIMMONS, J. S. (1926) A culture medium for differentiating organisms of the typhoid-colon-aerogenes groups and for isolating certain groups of fungi. *Journal of Infectious Disease*, **39**: 209.

**Further reading**

INTERNATIONAL COMMISSION ON MICROBIOLOGICAL SPECIFICATIONS FOR FOODS (ICMSF) (1978) *Microbial Ecology of Foods. Volume 2—Food Commodities*. London: Academic Press.

INTERNATIONAL COMMISSION ON MICROBIOLOGICAL SPECIFICATIONS FOR FOODS (ICMSF) (1988) *Micro-Organisms in Foods 2: Sampling for Microbiological Analysis: Principles and Specific Applications*. Oxford: Blackwell Scientific Publications.

## SALMONELLA

### Introduction

The presence of a member of the genus *Salmonella* in a food is indicative of poor handling. In view of the high risk of illnesses caused by the salmonellae, the pathogen should not be present in a food for human consumption.

### Rationale

The presence of *Salmonella* in fresh and dried food is indicative of poor hygienic practices during handling and processing. Generally, this pathogen has a zero tolerance rating, as its presence in a food means that there is a high risk to the consumer.

### Suitability

This method is suitable for detecting the presence of *Salmonella* in a range of fresh food and processed products. If, as in the case of fermented cassava, the sample has a low pH, an acid neutralizer, such as 1% calcium carbonate (w/v), should be added to the pre-enrichment broth.

### Limitations

This method should be carried out in an authorized laboratory by a trained operative. The test is for the presence or absence only.

### Principles

A sample of the test product is pre-enriched in a non-selective broth, to encourage recovery of damaged/stressed cells. After 16–20 h incubation, portions of broth are transferred to selective enrichment broths. Following a further incubation period, loopfuls of the selective enrichment broths are transferred to diagnostic agars. After 18–24 h incubation, characteristic colonies of *Salmonella* can be selected and confirmed by biochemical tests and serotyping.

### Requirements

#### Equipment

- Incubator at 35/37 °C
- Water-bath at 42 °C
- Water-bath at 50 °C
- Balance
- Waring blender and base

#### Consumables

- Autoclavable 500 ml bottles
- Pipettes, 1.0 ml
- Universal containers
- Petri dishes
- Containers for sterilizing Petri dishes
- Containers for sterilizing pipettes
- Buffered Peptone Water (Oxoid CM509)
- Rappaport Vassiliadis (RV) Enrichment Broth (Oxoid CM669)
- Selenite Cystine Broth (Oxoid CM699)
- Sodium biselenite (Oxoid LP121)
- Modified Brilliant Green Agar (Oxoid CM329)
- Bismuth Sulphite Agar (Oxoid CM201)

## SALMONELLA

- XLD Medium (Oxoid CM469)
- Triple Sugar Iron Agar (Oxoid CM277)
- Lysine Iron Agar (Oxoid CM381)
- Urea solution 40% (Oxoid SR020)
- Urea, Broth Base (Oxoid CM171)
- Saline, 0.85%
- Poly 'O' antigens
- Poly 'H' antigens
- Microscope slides
- Loops

### *Hazardous chemicals*

*Disodium phosphate:* may be harmful if inhaled, swallowed or absorbed through skin; irritant to eyes and skin; avoid breathing dust, avoid contact with skin or eyes.

*Ethanol:* may be harmful if inhaled, swallowed or absorbed through skin; irritant; avoid inhalation of vapour, avoid contact with skin and eyes; flammable; avoid heat, open flame or sparks.

*Ferric citrate:* may be harmful if inhaled, swallowed or absorbed through skin; may be irritant to eyes and skin; avoid inhalation of dust, avoid contact with skin or eyes.

*Ferrous sulphate:* may be harmful if inhaled, swallowed or absorbed through skin; irritant to eyes, skin and upper respiratory tract; avoid inhalation of dust, avoid contact with skin or eyes.

*Magnesium chloride 6 H<sub>2</sub>O:* may be harmful if inhaled, swallowed or absorbed through skin; irritant to eyes and skin and upper respiratory tract; avoid inhalation of dust, avoid contact with skin or eyes.

*Sodium chloride:* may be harmful if inhaled, ingested or absorbed through skin; irritant to skin, eyes and upper respiratory tract; avoid inhalation of dust, avoid contact with skin and eyes.

*Sodium thiosulphate:* may be harmful if inhaled, swallowed or absorbed through skin; irritant to eyes and skin; avoid breathing dust, avoid contact with skin or eyes.

*Sodium biselenite:* Very toxic by inhalation and if swallowed; causes severe burns; damage by cumulative effect; tetragenic, so handle with care.

Note: Microbiological media may contain potentially hazardous compounds. It is recommended that the manufacturer's instructions and warnings are followed closely.

## **Procedure**

### *Preparation of Buffered Peptone Water*

Formula (Oxoid)	
Peptone	10.0 g/l
Sodium chloride	5.0 g/l
Disodium phosphate	3.5 g/l
Potassium dihydrogen phosphate	1.5 g/l
Final pH	7.2 ± 0.2

Add 20 g to 1 l of distilled water. Mix well and distribute into final containers. Sterilize by autoclaving at 121 °C for 15 min.

### *Preparation of Selenite Cystine Broth*

Formula (Oxoid)	
Tryptone	5.0 g/l
Lactose	4.0 g/l

Disodium phosphate	10.0 g/l
L-cystine	0.01 g/l
Final pH	7.0 ± 0.2

Dissolve 4 g of sodium biselenite in 1 l of distilled water and then add the 19 g of Selenite Cystine Broth Base (CM699). Warm to dissolve and dispense into containers to a depth of at least 16 mm. Sterilize by placing in free-flowing steam for 15 min.

### *Preparation of Rappaport-Vassiliadis (RV) Enrichment Broth*

Formula (Oxoid)	
Soya Peptone	5.0 g/l
Sodium chloride	8.0 g/l
Potassium dihydrogen phosphate	1.6 g/l
Magnesium chloride 6 H <sub>2</sub> O	40.0 g/l
Malachite green	0.04 g/l
Final pH	5.2 ± 0.2

Weigh 30 g (the equivalent weight of dehydrated medium/l) and add to 1 l of distilled water. Heat gently until dissolved completely. Dispense 10 ml volumes into screw-capped bottles or tubes and sterilize by autoclaving at 115 °C for 15 min.

### *Preparation of Brilliant Green Agar (modified)*

Formula (Oxoid)	
'Lab-Lemco' Powder	5.0 g/l
Peptone	10.0 g/l
Yeast Extract	3.0 g/l
Disodium hydrogen phosphate	1.0 g/l
Sodium dihydrogen phosphate	0.6 g/l
Lactose	10.0 g/l
Sucrose	10.0 g/l
Phenol red	0.09 g/l
Brilliant green	0.7 g/l
Agar	12.0 g/l
Final pH	6.9 ± 0.2

Suspend 52 g in 1 l of distilled water. Heat gently with occasional agitation. Bring just to the boil and dissolve medium completely. DO NOT AUTOCLAVE. Cool to 50 °C, mix well and pour plates.

### *Preparation of Bismuth Sulphite Agar*

Formula (Oxoid)	
Peptone	5.0 g/l
'Lab-Lemco' Powder	5.0 g/l
Glucose	5.0 g/l
Disodium phosphate	4.0 g/l
Ferrous sulphate	0.3 g/l
Bismuth sulphite indicator	8.0 g/l
Brilliant green	0.016 g/l
Agar	12.7 g/l
Final pH	7.6 ± 0.2

Suspend 20 g in 500 ml of distilled water in a 1 l flask. Heat gently with frequent agitation until the medium just begins to boil and simmer for 30 s to dissolve the agar. Cool to 50–55 °C, mix well to disperse suspension and pour thick plates (25 ml medium/plate). Allow the medium to solidify with the dish uncovered. Larger volumes may be prepared if great care is taken and adequate head space provided. Store the plates for 3 days at 4 °C before use.

### *Preparation of XLD Medium*

Formula (Oxoid)	
Yeast Extract	3.0 g/l
L-lysine HCl	5.0 g/l
Xylose	3.75 g/l
Lactose	7.5 g/l

## SALMONELLA

Sucrose	7.5 g/l
Ferrous sulphate	0.8 g/l
Phenol red	0.08 g/l
Agar	12.5 g/l
Final pH	7.4 ± 0.2

Suspend the 53 g in 1 l of distilled water. Heat with frequent agitation until the medium boils. DO NOT OVERHEAT. Transfer immediately to a water-bath at 50 °C. Pour into plates as soon as the medium has cooled.

### *Preparation of Triple Sugar Iron Agar*

#### Formula (Oxoid)

'Lab-Lemco' Powder	3.0 g/l
Yeast Extract	3.0 g/l
Peptone	20.0 g/l
Sodium chloride	5.0 g/l
Lactose	10.0 g/l
Sucrose	10.0 g/l
Glucose	1.0 g/l
Ferric citrate	0.3 g/l
Sodium thiosulphate	0.3 g/l
Phenol red	0.02 g/l
Agar	12.0 g/l
Final pH	7.4 ± 0.2

Suspend the 65 g in 1 l of distilled water. Bring to the boil to dissolve completely. Mix well and distribute. Sterilize by autoclaving at 121 °C for 15 min. Allow the medium to set in sloped form with a butt about 3 cm deep.

### *Preparation of Lysine Iron Agar*

#### Formula (Oxoid)

Bacteriological Peptone	5.0 g/l
Yeast Extract	3.0 g/l
Glucose	1.0 g/l
L-lysine	10.0 g/l
Ferric ammonium citrate	0.5 g/l
Sodium thiosulphate	0.04 g/l
Bromocresol purple	0.02 g/l
Agar	14.5 g/l
Final pH	6.7 ± 0.2

Suspend the 34 g in 1 l of distilled water. Bring to the boil to dissolve completely. Dispense into tubes and sterilize by autoclaving at 121 °C for 15 min. Cool the tubes in an inclined position to form slants with deep butts.

### *Preparation of Urea Broth*

#### Formula (Oxoid)

Peptone	1.0 g/l
Glucose	1.0 g/l
Disodium phosphate	1.2 g/l
Potassium dihydrogen phosphate	0.8 g/l
Sodium chloride	5.0 g/l
Phenol red	0.004 g/l
Final pH	6.8 ± 0.2

Add 0.9 g to 95 ml of distilled water. Sterilize by autoclaving at 115 °C for 20 min. Cool to 55 °C and aseptically introduce 5 ml of sterile 40% Urea Solution (Oxoid SR 200). Mix well and distribute 10 ml amounts into sterile containers.

### *Isolation of Salmonella*

Add 25 g of sample to a 500 ml wide-necked vessel containing 225 ml of Buffered Peptone Water (BPW). If the sample does not disperse readily in the BPW, blend in a mechanical blender. Incubate for 16–20 h at 35 °C or 37 °C.

Pipette 1 ml of the pre-enrichment broth into 10 ml Selenite Cystine Broth (SC) and 0.1 ml into 10 ml Rappaport-Vassiliadis Broth (RV). Incubate the RV broth in a water-bath at 42 °C for 24 h and the SC broth at 35 °C or 37 °C for 24 and 48 h.

Transfer a loopful of each of the two selective enrichment broths onto the surface of each of three selective agar media: Brilliant Green Agar, Bismuth Sulphite Agar and a 'laboratory choice' (e.g. XLD, Desoxycholate Citrate Agar, Salmonella-Shigella agar or Hektoen Enteric Agar). Incubate plates at 35 °C or 37 °C and observe for characteristic colonies of *Salmonella* after 24 and 48 h.

### Controls

Positive control: *Salmonella typhimurium*

Negative control: *Escherichia coli*

### Analysis of results

Pick, with a sterile inoculating needle, two or more of each suspect colony type from the Brilliant Green Agar, Bismuth Sulphite Agar and 'laboratory choice' agar. Transfer to a nutrient agar plate, to check the purity of the culture, and stab and streak slopes of Triple Sugar Iron Agar (TSI) and Lysine Iron Agar (LIA). Incubate slopes at 35 °C or 37 °C for 24 h. Record reactions as described below.

**Table 3.16 Reactions on Lysine Iron Agar**

Cultures	Slant	Butt	H <sub>2</sub> S
<i>Arizona</i>	Alkaline (purple)	Alkaline	+
<i>Salmonella</i>	Alkaline	Alkaline	+
<i>Proteus</i>	Red	Acid (yellow)	–
<i>Providencia</i>	Red	Acid	–
<i>Citrobacter</i>	Alkaline	Acid	+
<i>Escherichia</i>	Alkaline	Acid or neutral	–
<i>Shigella</i>	Alkaline	Acid	–
<i>Klebsiella</i>	Alkaline	Alkaline	–

**Table 3.17 Reactions on Triple Sugar Iron Agar**

Organism	Butt	Slope	H <sub>2</sub> S
<i>Aerobacter aerogenes</i>	AG	A	–
<i>Aerobacter cloacae</i>	AG	A	–
<i>Escherichia coli</i>	AG	A	–
<i>Proteus vulgaris</i>	AG	A	+
<i>Proteus morganii</i>	A or G	NC or ALK	–
<i>Shigella dysenteriae</i>	A	NC or ALK	–
<i>Shigella sonnei</i>	A	NC or ALK	–
<i>Salmonella typhosa</i>	A	NC or ALK	+
<i>Salmonella paratyphi</i>	AG	NC or ALK	–
<i>Salmonella enteritidis</i>	AG	NC or ALK	+
<i>Salmonella typhimurium</i>	AG	NC or ALK	+

Key: AG = acid (yellow) and gas formation

A = acid (yellow)

NC = no change

ALK = alkaline (red)

+ = hydrogen sulphide (black)

– = no hydrogen sulphide produced

Cultures which give positive reactions for *Salmonella* can be further identified by serological tests, using poly-O and poly-H antigens.

An additional test for the differentiation of *Salmonella* and *Shigella* species from other members of the Enterobacteriaceae is the production of urease. Inoculate Urea Broth with a pure culture of the suspect bacterium. Incubate at 35 °C and examine after 6 h. Any organism producing a pink colour in the broth (urease positive) does not belong to the *Salmonella/Shigella* group and can be discarded.

### International standards

Codex (CAC/RCP 1–1969; Rev. 2–1985) states that fresh produce and processed foods should be free from micro-organisms which may represent a hazard to health.

## References

### Source

INTERNATIONAL COMMISSION ON MICROBIOLOGICAL SPECIFICATIONS FOR FOODS (ICMSF) (1978) *Micro-Organisms in Foods. 1. Their Significance and Methods of Enumeration*, 2nd edn. Toronto, Canada: University of Toronto Press.

CODEX CAC/RCP 1–1969; REV. 2–1985. Codex Alimentarius Commission, FAO/WHO CX/CPL 92/9 June 1992.

### Further reading

EDWARDS, P. R. and FIFE, M. A. (1961) Lysine-iron agar in the detection of *Arizona* cultures. *Applied Microbiology*, **9**: 478.

HYNES, M. (1942) The isolation of intestinal pathogens by selective media. *Journal of Pathology and Bacteriology*, **54**: 193.

TAYLOR, W. I. (1965) Isolation of *Shigella*. 1. Xylose lysine agars; new media for isolation of enteric pathogens. *American Journal of Clinical Pathology*, **44**: 471.

## STAPHYLOCOCCUS AUREUS

### Introduction

#### Rationale

The presence of *Staphylococcus aureus* in fresh and dried food is indicative of poor hygiene practices during handling and processing. If the pathogen is present in a sample at levels of  $10^4$  cfu/g or above, the production of a heat-stable toxin may occur.

#### Suitability

This method is suitable for determining the level of *S. aureus* in a range of fresh, fermented and processed products. It is important that a representative sample is taken and that at least two or preferably five sub-samples are tested from each batch.

#### Limitations

This method should be carried out in an authorized laboratory by a trained operative.

#### Principles

The sample is mixed together with a sterile diluent, from which a 10-fold dilution series is prepared. Aliquots from a range of dilutions are transferred onto a diagnostic culture medium and the plates are incubated under aerobic conditions at 35 °C. After the required incubation period, the number of characteristic colonies of *S. aureus* is counted and the level of the pathogen present in the sample is expressed as colony forming units (cfu)/g.

### Requirements

#### Equipment

- Incubator at 35 °C
- Autoclave
- Balance
- Waring blender and base
- Colony counter

#### Consumables

- Petri dishes
- Containers for sterilizing Petri dishes
- Glass bottles with autoclavable lids

- Autoclave tape
- Pipettes, 1.0 ml, blow-out
- Containers for sterilizing pipettes
- Universal containers
- Maximum Recovery Diluent (Oxoid CM733)
- Baird-Parker Medium (Oxoid CM275)
- Egg Yolk Tellurite Emulsion (Oxoid SR054)
- Rabbit plasma
- Alcohol
- Glass beaker
- Glass 'hockey sticks'

### *Hazardous chemicals*

*Ethanol:* may be harmful if inhaled, swallowed or absorbed through skin; irritant; avoid inhalation of vapour, avoid contact with skin and eyes; flammable; avoid heat, open flame or sparks.

*Lithium chloride:* may be harmful if inhaled, swallowed or absorbed through skin; irritant to eyes, skin and upper respiratory tract; use in a fume hood; avoid inhalation of dust, and contact with skin or eyes.

Note: Microbiological media may contain potentially hazardous compounds. It is recommended that the manufacturer's instructions and warnings are followed closely.

## **Procedure**

### *Preparation of Baird-Parker Agar*

Formula (Oxoid)	
Tryptone	10.0 g/l
'Lab-Lemco' Powder	5.0 g/l
Yeast Extract	1.0 g/l
Sodium pyruvate	10.0 g/l
Glycine	12.0 g/l
Lithium chloride	5.0 g/l
Agar	20.0 g/l
Final pH	6.8 ± 0.2

Suspend the 63 g in 1 l of distilled water and heat gently until the agar is completely dissolved. Sterilize by autoclaving at 121 °C for 15 min. Allow the medium to cool to 50 °C. Aseptically add 50 ml of Egg Yolk Tellurite Emulsion, mix well and pour into sterile Petri dishes.

### *Enumeration of Staphylococcus aureus*

Prepare a 10-fold dilution series as described in *Preparation of dilution series, Aerobic plate count*, above. Inoculate duplicate plates with 0.1 ml of each dilution and spread over the surface using a sterile glass 'hockey stick'. Incubate plates at 35 °C for 18–24 h.

### *Confirmation of Staphylococcus aureus—coagulase test*

Culture presumptive colonies of *S. aureus* onto Nutrient Agar and incubate overnight at 35 °C. Gram stain to confirm the presence of Gram-positive cocci. Place a drop of sterile distilled water on a 3 × 1 in microscope slide. Emulsify the colony under test. Add one drop of rabbit plasma, reconstituted with 2 ml sterile distilled water, and mix well. Clumping within 10 s indicates the presence of coagulase-positive staphylococci.

### *Controls*

Positive control: *Staphylococcus aureus*



## STAPHYLOCOCCUS AUREUS

Negative control: *Escherichia coli*

### Analysis of results

After 18–24 h of incubation, count the number of colonies which are black and shiny with narrow white margins and surrounded by clear zones. There is a high probability that these are colonies of *S. aureus*. Mark the position of these colonies and incubate for a further 18 h.

At the end of the reincubation period, count all colonies with the appearance described above and, in addition, colonies which are shiny black with or without narrow white margins and without clear zones. Pick off a minimum of five colonies from each plate and test for coagulase production.

The number of characteristic colonies on Baird-Parker Agar which produce clear zones after 30 h should be added to the proportion which were coagulase-positive after the extended incubation period.

The mean of the duplicate plates should be multiplied by 10, to give the number of cfu/ml dilution. This number is then multiplied by the dilution factor to give the number of cfu/g sample.

### International standards

There are no known International Standards for NGSS.

### References

#### Source

BRIDSON, E. Y. (comp.) (1990) *Oxoid Manual*. 6th edn. Basingstoke, UK: Unipath Ltd.

INTERNATIONAL COMMISSION ON MICROBIOLOGICAL SPECIFICATIONS FOR FOODS (ICMSF) (1978) *Micro-Organisms in Foods. 1. Their Significance and Methods of Enumeration*. 2nd edn. Toronto, Canada: University of Toronto Press.

#### Further reading

HARRIGAN, W. F. and McCANCE, M. E. (1976) *Laboratory Methods in Food and Dairy Microbiology*. London: Academic Press.

INTERNATIONAL COMMISSION ON MICROBIOLOGICAL SPECIFICATIONS FOR FOODS (ICMSF) (1978) *Microbial Ecology of Foods. Volume 2—Food Commodities*. London: Academic Press.

INTERNATIONAL COMMISSION ON MICROBIOLOGICAL SPECIFICATIONS FOR FOODS (ICMSF) (1988) *Micro-Organisms in Foods 2: Sampling for Microbiological Analysis: Principles and Specific Applications*. Oxford: Blackwell Scientific Publications.

## CLOSTRIDIUM PERFRINGENS

### Introduction

#### Rationale

The presence of high levels ( $>10^6$  cfu/g) of the spore-forming pathogen *Clostridium perfringens* in fresh and dried food is a potential hazard (ICMSF, 1978).

#### Suitability

This method is suitable for determining the level of *C. perfringens* in a range of fresh, fermented and processed products. It is important that a representative sample is taken and that at least five sub-samples are tested from each batch.

#### Limitations

This method should be carried out in an authorized laboratory by a trained operative.

#### Principles

The sample is mixed together with a sterile diluent, from which a ten-fold dilution series is prepared. Aliquots from a range of dilutions are transferred onto a diagnostic culture medium and the plates are incubated under anaerobic conditions at 35 °C.

After the required incubation period, the number of characteristic colonies of *C. perfringens* is counted and the level of the pathogen present in the sample is expressed as colony-forming units (cfu)/g.

*Clostridium botulinum* produces one of the most highly toxic substances known to man. *C. botulinum* will grow on the media described for the enumeration of *C. perfringens*. Suspect cultures of *C. botulinum* should be taken to the nearest reference laboratory for confirmation.

## Requirements

### Equipment

- Incubator at 35 °C
- Autoclave
- Balance
- Waring blender and base
- Colony counter
- Anaerobic jar

### Consumables

- Petri dishes
- Containers for sterilizing Petri dishes
- Glass bottles with autoclavable lids
- Autoclave tape
- Pipettes, 1.0 ml, blow-out
- Containers for sterilizing pipettes
- Universal containers
- Maximum Recovery Diluent (Oxoid CM733)
- Perfringens Agar Base (Oxoid CM587)
- Perfringens (SFP) Selective Supplement (Oxoid SR93)
- Egg Yolk Emulsion (Oxoid SR47)
- Beef Extract
- Peptone
- Potassium nitrate
- Galactose
- Agar
- Tryptose
- Yeast Extract
- Sodium monohydrogen phosphate
- Phenol red
- Gelatin
- Alcohol
- Glass beaker
- Glass 'hockey sticks'
- Anaerobic gas packs

## CLOSTRIDIUM PERFRINGENS

### *Hazardous chemicals*

- Ethanol:** *may be harmful if inhaled, swallowed or absorbed through skin; irritant; avoid inhalation of vapour, and contact with skin or eyes; flammable; avoid heat, open flame or sparks.*
- Ferric ammonium citrate:** *may be harmful if inhaled, swallowed or absorbed through skin; may irritate eyes and skin; avoid inhalation of dust, avoid contact with skin or eyes.*
- Lithium chloride:** *may be harmful if inhaled, swallowed or absorbed through skin; irritant to eyes, skin and upper respiratory tract; use in a fume hood; avoid inhalation of dust, and contact with skin or eyes.*
- Potassium nitrate:** *may be harmful if inhaled, swallowed or absorbed through skin; irritant to eyes, skin and upper respiratory tract; use in a fume hood; avoid inhalation of dust, and contact with skin or eyes.*
- Sodium metabisulphite:** *may be harmful if inhaled, swallowed or absorbed through skin; may be irritant to eyes and skin; avoid inhalation of dust, avoid contact with skin or eyes.*
- Sodium monohydrogen phosphate:** *may be harmful if inhaled, swallowed or absorbed through skin; irritant to eyes and skin; avoid inhalation of dust, avoid contact with skin or eyes.*

Note: Microbiological media may contain potentially hazardous compounds. It is recommended that the manufacturer's instructions and warnings are followed closely.

### **Procedure**

#### *Preparation of Shahidi-Ferguson Perfringens Agar (SFP Agar)*

Formula (Oxoid)

Tryptone	15.0 g/l
Soya Peptone	5.0 g/l
'Lab-Lemco' Powder	5.0 g/l
Yeast Extract	5.0 g/l
Sodium metabisulphite	1.0 g/l
Ferric ammonium citrate	1.0 g/l
Agar	14.0 g/l
Final pH	7.6 ± 0.2

Suspend 23 g in 500 ml of distilled water and heat gently until the agar is completely dissolved. Sterilize by autoclaving at 121 °C for 10 min. Allow the medium to cool to 50 °C. Add the rehydrated contents of one vial of the SFP supplement and 25 ml of Egg Yolk Emulsion, mix well and pour into sterile Petri dishes.

#### *Enumeration of Clostridium perfringens*

Prepare a 10-fold dilution series as described in *Preparation of dilution series, Aerobic plate count*, above. Inoculate duplicate plates with 0.1 ml of each dilution and spread over the surface using a sterile glass 'hockey stick'. Overlay with an additional 10 ml of egg yolk-free SFP Agar. Incubate plates at 35 °C for 18–24 h in an anaerobic jar and gas pack. Count the number of characteristic colonies. *C. perfringens* colonies appear as large black colonies.

#### *Preparation of Motility-Nitrate Medium*

Formula

Beef Extract	3.0 g/l
Peptone	5.0 g/l
Potassium nitrate	1.0 g/l
Galactose	5.0 g/l
Glycerol	5.0 g/l
Agar	3.0 g/l

Dissolve ingredients in distilled water, dispense in 13 ml volumes into screw-capped tubes and autoclave at 121 °C for 15 min. If this medium is not used the same day, it must be de-aerated before inoculation; open the tubes slightly, keep in a boiling water-bath for 10 min close, cool until agar resolidifies.

### Preparation of Lactose Gelatin

#### Formula

Tryptose	15.0 g/l
Yeast Extract	10.0 g/l
Lactose	10.0 g/l
Sodium monohydrogen phosphate	5.0 g/l
Phenol red (1% solution)	5.0 ml
Gelatin	120.0 g/l

Dissolve the ingredients, except the gelatin, in 1000 ml distilled water, adjust the pH to 7.5, then add the gelatin and heat to dissolve. Dispense in 13 ml volumes into screw-capped bottles and autoclave at 121 °C for 15 min. If the medium is not used on the same day, it should be de-aerated as described in *Preparation of Motility-Nitrate Medium* above.

### Confirmation of presumptive *Clostridium perfringens*

Stab-inoculate five black colonies into the Motility-Nitrate Medium. Inoculate from the same colonies into Lactose Gelatin. Close the tubes tightly and incubate at 35–37 °C for 24 h. Add 0.1 ml of nitrite test reagent to each tube of Motility-Nitrate Broth. Production of a pink or red colour indicates the presence of nitrite.

Place the Lactose Gelatin tubes in iced water for 10 min, observe and record liquefaction. If liquefaction has not occurred, but the culture is non-motile and positive for nitrite production, reincubate the Lactose Gelatin tube for a further 24 h. Calculate the confirmed *C. perfringens* count from the presumptive count and the ratio of confirmed colonies to the total number of colonies tested.

### Controls

Positive control: *Clostridium perfringens*

Negative control: *Staphylococcus aureus*

### Analysis of results

#### Enumeration of bacteria

The number of colonies on SFP Agar should be counted after 18–24 h. Counts should be made from plates supporting 30–300 colonies and the mean of the duplicate plates noted. The mean should be multiplied by 10 to give the number of cfu/ml dilution. This number is then multiplied by the dilution factor to give the number of cfu/g sample. The level of *C. perfringens* should be expressed as the number of colony forming units/g of sample (ICMSF, 1978).

### International standards

There are no known International Standards for NGSS.

### References

#### Source

BRIDSON, E. Y. (comp.) (1990) *Oxoid Manual*, 6th edn. Basingstoke, UK: Unipath Ltd.

INTERNATIONAL COMMISSION ON MICROBIOLOGICAL SPECIFICATIONS FOR FOODS (ICMSF) (1978) *Micro-Organisms in Foods. Volume 1. Their Significance and Methods of Enumeration*, 2nd edn. Toronto, Canada: University of Toronto Press.

#### Further reading

HARRIGAN, W. F. and McCANCE, M. E. (1976) *Laboratory Methods in Food and Dairy Microbiology*. London: Academic Press.

INTERNATIONAL COMMISSION ON MICROBIOLOGICAL SPECIFICATION FOR FOODS (ICMSF) (1978) *Micro-Organisms in Foods. Volume 1. Their Significance and Methods of Enumeration*, 2nd edn, Toronto, Canada: University of Toronto Press.

INTERNATIONAL COMMISSION ON MICROBIOLOGICAL SPECIFICATIONS FOR FOODS (ICMSF) (1978) *Microbial Ecology of Foods. Volume 2. Food Commodities*. London: Academic Press.

INTERNATIONAL COMMISSION ON MICROBIOLOGICAL SPECIFICATIONS FOR FOODS (ICMSF) (1988) *Micro-Organisms in Foods. Volume 2. Sampling for Microbiological Analysis: Principles and Specific Applications*. Oxford: Blackwell Scientific Publications.

## BACILLUS CEREUS

### *BACILLUS CEREUS*

#### **Introduction**

A plate count for *Bacillus cereus* provides information on the level of this pathogen in a sample.

#### *Rationale*

*B. cereus* produces heat-resistant bodies called spores, which can withstand standard cooking procedures. If the pathogen reaches high levels in a food during production, toxin may be produced. If the food is then heated gently, the toxin may survive and cause food poisoning after consumption.

#### *Suitability*

This method is suitable for determining the level of *B. cereus* in a wide range of dried products.

#### *Limitations*

This method should only be carried out in the laboratory by a trained operative.

#### *Principle*

A 10-fold dilution series is prepared from the sample and plated onto a diagnostic agar. Following incubation for 24/48 h, a confirmatory staining procedure is made on suspect colonies of *B. cereus*. The number of suspect colonies is confirmed and the level of the pathogen is reported as colony forming units/g sample.

#### **Requirements**

##### *Equipment*

- Incubator at 35 °C
- Autoclave
- Balance
- Waring blender and base

##### *Consumables*

- Containers for sterilizing Petri dishes
- Glass bottles with autoclavable lids
- Pipettes, 1.0 ml, blow-out
- Containers for sterilizing pipettes
- Universal containers
- *B. cereus* Agar (Oxoid CM617)
- *B. cereus* Selective Supplement (Oxoid SR99)
- Egg Yolk Emulsion (Oxoid SR47)
- Malachite green
- Sudan black
- Alcohol
- Xylene
- Safranin
- Microscope slides
- Forceps
- Bunsen burner

- Loops
- Saline solution (0.85% NaCl)

### Hazardous chemicals

<i>Ethanol:</i>	<i>may be harmful if inhaled, swallowed or absorbed through skin; irritant; avoid inhalation of vapour, and contact with skin or eyes; flammable; avoid heat, open flame or sparks.</i>
<i>Disodium hydrogen phosphate:</i>	<i>may be harmful if inhaled, swallowed or absorbed through skin; irritant to eyes and skin; avoid inhalation of dust, avoid contact with skin or eyes.</i>
<i>Magnesium sulphate:</i>	<i>may be harmful if inhaled, swallowed or absorbed through skin; irritant to eyes; avoid inhalation of dust.</i>
<i>Potassium dihydrogen phosphate:</i>	<i>may be harmful if inhaled, swallowed or absorbed through skin; irritant to eyes and skin; avoid inhalation of dust, avoid contact with skin or eyes.</i>
<i>Sodium chloride:</i>	<i>may be harmful if inhaled, swallowed or absorbed through skin; irritant to eyes and skin; avoid inhalation of dust, avoid contact with skin or eyes.</i>

Note: Microbiological media may contain potentially hazardous compounds. It is recommended that the manufacturer's instructions and warnings are followed closely.

## Procedure

### Preparation of *B. cereus* Agar

Formula (Oxoid)

Peptone	1.0 g/l
Mannitol	10.0 g/l
Sodium chloride	2.0 g/l
Magnesium sulphate	0.1 g/l
Disodium hydrogen phosphate	2.5 g/l
Potassium dihydrogen phosphate	0.25 g/l
Bromothymol blue	0.12 g/l
Sodium pyruvate	10.0 g/l
Agar	14.0 g/l

Final pH 7.2 ± 0.2

*B. cereus* Selective Supplement, Oxoid Code: SR99. Vial contents (each vial is sufficient for 500 ml of medium)  
Polymyxin B 50 000 IU

Suspend 20.5 g in 475 ml of distilled water and bring gently to the boil to dissolve completely. Sterilize by autoclaving at 121 °C for 15 min. Cool to 50 °C and aseptically add the contents of one vial of *B. cereus* Selective Supplement SR99 reconstituted with 2 ml of sterile distilled water. Then add 25 ml of sterile Egg Yolk Emulsion Oxoid, SR47, mix well and pour into sterile Petri dishes.

### Enumeration of *Bacillus cereus* (Oxoid method)

Prepare food sample as described in *Preparation of dilution series, Aerobic plate count*, above. Pipette 0.1 ml each of the decimal dilutions onto duplicate sets of pre-poured plates of *B. cereus* Agar (Oxoid). Incubate plates at 37 °C for 24 h.

### Controls

Positive control: *Bacillus cereus*

Negative control: *Staphylococcus aureus*

### Analysis of results

Following incubation, examine plates for typical colonies of *B. cereus*. Confirm this presumptive identification of *B. cereus* by using the rapid confirmatory staining procedure (see *Rapid confirmatory staining procedure* below). Report the results as the number of *B. cereus* colonies/g of food sample.

## BACILLUS CEREUS

### *Rapid confirmatory staining procedure*

Prepare films from the centre of a 1 day-old colony or from the edge of a 2-day colony. Air dry the film and fix with minimal flaming.

Place the slide over boiling water and flood with 5% w/v malachite green. After 2 min wash and blot dry the slide. Stain with 0.3% w/v Sudan black in 70% ethanol for 15 min. Wash the slide with xylene for 5 s and blot dry. Counterstain with 0.5% w/v Safranin for 20 s. Spores stain pale to mid-green, central and no swelling of cells. Lipid globules stain black and cytoplasm stains red.

### **International standards**

There are no known standards for NGSS.

### **References**

#### *Source*

ANON. (1982) *Oxoid Manual*. 5th edn. Basingstoke, UK: Oxoid Ltd.

BRIDSON, E. Y. (comp.) (1990) *Oxoid Manual*. 6th edn. Basingstoke, UK: Unipath Ltd.

#### *Further reading*

INTERNATIONAL COMMISSION ON MICROBIOLOGICAL SPECIFICATIONS FOR FOODS (ICMSF) (1978) *Micro-Organisms in Foods. Volume 1. Their Significance and Methods of Enumeration*. 2nd edn. Toronto: University of Toronto Press.

## LACTIC ACID BACTERIA

### **Introduction**

#### *Rationale*

The lactic acid bacteria are members of genera in which lactic acid is the major end-product of carbohydrate catabolism. This method can be used to monitor the levels of lactic acid bacteria in a traditional fermented product.

#### *Suitability*

This method is suitable for determining the level of lactic acid bacteria in a range of fresh, fermented and processed products. It is important that a representative sample is taken and that at least five sub-samples are tested from each batch.

#### *Limitations*

This method should be carried out in an authorized laboratory by a trained operative.

#### *Principle*

The sample is mixed together with a sterile diluent from which a 10-fold dilution series is prepared. Aliquots from a range of dilutions are transferred onto a diagnostic culture medium and the plates are incubated under micro-aerophilic conditions at 30 °C. After the required incubation period, the number of characteristic colonies of lactic acid bacteria is counted and the level is expressed as the number of colony forming units (cfu)/g.

### **Requirements**

#### *Equipment*

- Incubator at 30 °C
- Autoclave
- Balance

- Waring blender and base
- Colony counter
- Anaerobic jar

### *Consumables*

- Petri dishes
- Containers for sterilizing Petri dishes
- Glass bottles with autoclavable lids
- Autoclave tape
- Pipettes, 1.0 ml, blow-out
- Containers for sterilizing pipettes
- Universal containers
- Maximum Recovery Diluent (Oxoid CM733)
- MRS Agar (Oxoid CM361)
- Hydrogen peroxide, 20 volume
- Alcohol
- Glass beaker
- Glass 'hockey sticks'
- Carbon dioxide gas packs

### *Hazardous chemicals*

*Dipotassium hydrogen phosphate: may be harmful if inhaled, swallowed or absorbed through skin; irritant to eyes and skin; avoid inhalation of dust, avoid contact with skin or eyes.*

*Ethanol: may be harmful if inhaled, swallowed or absorbed through skin; irritant; avoid inhalation of vapour, and contact with skin or eyes; flammable; avoid heat, open flame or sparks.*

*Magnesium sulphate, 7H<sub>2</sub>O: may be harmful if inhaled, swallowed or absorbed through skin; irritant to eyes; avoid inhalation of dust, avoid contact with eyes or skin.*

*Manganese sulphate, 4H<sub>2</sub>O: may be harmful if inhaled, swallowed or absorbed through skin; irritant to eyes; avoid inhalation of dust, avoid contact with eyes or skin.*

*Sodium acetate, 3H<sub>2</sub>O: may be harmful if inhaled, swallowed or absorbed through skin; avoid inhalation of dust, avoid contact with skin or eyes.*

*Triammonium citrate: may be harmful if inhaled, swallowed or absorbed through skin; irritant to eyes skin and upper respiratory tract; avoid inhalation of dust, avoid contact with skin or eyes.*

Note: Microbiological media may contain potentially hazardous compounds. It is recommended that the manufacturer's instructions and warnings are followed closely.

### **Procedure**

#### *Preparation of de Man, Rogosa and Sharpe Agar (MRS Agar)*

##### Formula

Peptone	10.0 g/l
'Lab-Lemco' Powder	8.0 g/l
Yeast Extract	4.0 g/l
Glucose	20.0 g/l
Sorbitan mono-oleate	1.0 ml



## LACTIC ACID BACTERIA

Dipotassium hydrogen phosphate	2.0 g/l
Sodium acetate, 3H <sub>2</sub> O	5.0 g/l
Triammonium citrate	2.0 g/l
Magnesium sulphate, 7H <sub>2</sub> O	0.2 g/l
Manganese sulphate, 4H <sub>2</sub> O	0.05 g/l
Agar	10.0 g/l
Final pH 6.2 + 0.2	

Suspend the 62 g in 1 l of distilled water. Boil to dissolve the medium completely. Dispense into tubes, bottles or flasks and sterilize by autoclaving at 121 °C for 15 min.

### *Enumeration of lactic acid bacteria*

Prepare a 10-fold dilution series as described in *Preparation of dilution series, Aerobic plate count*, above. Inoculate duplicate plates with 0.1 ml of each dilution and spread over the surface using a sterile glass 'hockey stick'. Incubate plates at 30 °C for 72 h in an anaerobic jar with a carbon dioxide gas pack.

### *Catalase test*

Perform stain from colonies first. If Gram-positive, carry out the catalase test. Flood the plate with enough 20 volume hydrogen peroxide to submerge the colonies. Catalase-positive colonies will effervesce.

## Analysis of results

### *Enumeration of lactic acid bacteria*

The number of characteristic colonies on MRS Agar should be counted after 72 h. Counts should be made from plates supporting 30–300 colonies and the mean of the two plates noted. The presence of presumptive lactic acid bacteria is confirmed by checking that characteristic colonies are catalase-negative, Gram-positive rods or cocci. The mean should be multiplied by 10 to give the number of cfu/ml dilution. This number is then multiplied by the dilution factor to give the number of cfu/g sample. The level of lactic acid bacteria should be expressed as the number of colony-forming units/g of sample (ICMSF, 1978).

## International standards

There are no known International Standards for NGSS.

## References

### *Source*

BRIDSON, E. Y. (comp.) (1990) *Oxoid Manual*. 6th edn. Basingstoke, UK: Unipath Ltd.

INTERNATIONAL COMMISSION ON MICROBIOLOGICAL SPECIFICATIONS FOR FOODS (ICMSF) (1978) *Micro-organisms in Foods. Volume 1. Their Significance and Methods of Enumeration*. 2nd edn. Toronto, Canada: University of Toronto Press.

### *Further reading*

HARRIGAN, W. F. and McCANCE, M. E. (1976) *Laboratory Methods in Food and Dairy Microbiology*. London: Academic Press.

INTERNATIONAL COMMISSION ON MICROBIOLOGICAL SPECIFICATIONS FOR FOODS (ICMSF) (1978) *Microbial Ecology of Foods. Volume 2. Food Commodities*. London: Academic Press.

INTERNATIONAL COMMISSION ON MICROBIOLOGICAL SPECIFICATIONS FOR FOODS (ICMSF) (1988) *Micro-Organisms in Foods, Volume 2. Sampling for Microbiological Analysis: Principles and Specific Applications*. Oxford: Blackwell Scientific Publications.

## YEAST AND MOULD COUNT

### Introduction

#### *Rationale*

Moulds and yeasts present in a sample can produce premature spoilage, and possible production of mycotoxins, if a product is not stored under the correct conditions.

### *Suitability*

This method is suitable for assessing the level of yeasts and moulds in a range of fresh, fermented and processed products. It is important that a representative sample is taken, and that at least five sub-samples are tested from each batch.

### *Limitations*

This method should be carried out in an authorized laboratory by a trained operative.

### *Principle*

The sample is mixed together with a sterile diluent from which a 10-fold dilution series is prepared. Aliquots from a range of dilutions are transferred onto a diagnostic culture medium and the plates are incubated under aerobic conditions at 25–30 °C. After the required incubation period, the number and types of characteristic colonies of yeasts and moulds can be noted and counted and the level expressed as colony forming units (cfu)/g.

## **Requirements**

### *Equipment*

- Incubator at 25–30 °C
- Autoclave
- Balance
- Waring blender and base
- Colony counter

### *Consumables*

- Petri dishes
- Containers for sterilizing Petri dishes
- Glass bottles with autoclavable lids
- Autoclave tape
- Pipettes, 1.0 ml, blow-out
- Containers for sterilizing pipettes
- Universal containers
- Bacteriological Peptone
- DRBC Agar Base (Oxoid CM727)
- Chloramphenicol Supplement (Oxoid SR78)
- Alcohol
- Glass beaker
- Glass 'hockey sticks'

### *Hazardous chemicals*

*Ethanol:* *may be harmful if inhaled, swallowed or absorbed through skin; irritant; avoid inhalation of vapour, and contact with skin or eyes; flammable; avoid heat, open flame or sparks.*

*Potassium dihydrogen phosphate:* *may be harmful if inhaled, swallowed or absorbed through skin; irritant to eyes and skin; avoid inhalation of dust and contact with skin or eyes.*

*Magnesium sulphate:* *may be harmful if inhaled, swallowed or absorbed through skin; irritant to eyes; avoid inhalation of dust, avoid contact with eyes and skin.*

Note: Microbiological media may contain potentially hazardous compounds. It is recommended that the manufacturer's instructions and warnings are followed closely.

**Procedure***Preparation of Dichloran Rose-Bengal Chloramphenicol Agar (DRBC)*

## Formula

Peptone	5.0 g/l
Glucose	10.0 g/l
Potassium dihydrogen phosphate	1.0 g/l
Magnesium sulphate	0.5 g/l
Dichloran	0.002 g/l
Rose-Bengal	0.025 g/l
Agar	15.0 g/l
Final pH	5.6 ± 0.2

Suspend 15.75 g in 500 ml of distilled water and heat gently until the agar is completely dissolved. Rehydrate one vial of Chloramphenicol Supplement SR78 and add to the DRBC Agar base. Sterilize by autoclaving at 121 °C for 15 min. Allow the medium to cool to 50 °C, mix well and pour into sterile Petri dishes.

*Preparation of 0.1% peptone*

Dissolve 1 g Bacteriological Peptone in 1 l distilled water. Dispense in required volumes (270 and 9.0 ml) and sterilize by autoclaving at 121 °C for 15 min.

*Enumeration of yeasts and moulds*

Prepare a 10-fold dilution series as described in *Preparation of dilution series, Aerobic plate count*, above, using 0.1% peptone as the diluent. Inoculate duplicate plates with 0.1 ml of each dilution and spread over the surface using a sterile glass 'hockey stick'. Incubate plates at 25–30 °C for 5–7 days.

**Analysis of results***Enumeration of yeasts and moulds*

The number of colonies on DRBC Agar should be counted after 5–7 days. Counts should be made from plates supporting 30–300 colonies and the mean of the two plates noted. The mean should be multiplied by 10 to give the number of cfu/ml dilution. This number is then multiplied by the dilution factor to give the number of cfu/g sample. The level of yeasts and moulds should be expressed as the number of colony-forming units/g of sample (ICMSF, 1978). The types and percentage of particular groups should also be noted.

**International standards**

There are no known International Standards for NGSS.

**References***Source*

BRIDSON, E. Y. (comp.) *Oxoid Manual*. 6th edn, Basingstoke, UK: Unipath Ltd.

INTERNATIONAL COMMISSION ON MICROBIOLOGICAL SPECIFICATIONS FOR FOODS (ICMSF) (1978) *Micro-Organisms in Foods. Volume 1. Their Significance and Methods of Enumeration*, 2nd edn. Toronto, Canada: University of Toronto Press.

*Further reading*

HARRIGAN, W. F. and McCANCE, M. E. (1976) *Laboratory methods in Food and Dairy Microbiology*. London: Academic Press.

INTERNATIONAL COMMISSION ON MICROBIOLOGICAL SPECIFICATIONS FOR FOODS (ICMSF) (1978) *Microbial Ecology of Foods. Volume 2—Food Commodities*. London: Academic Press.

INTERNATIONAL COMMISSION ON MICROBIOLOGICAL SPECIFICATIONS FOR FOODS (ICMSF) (1988) *Micro-Organisms in Foods. Volume 2: Sampling for Microbiological Analysis: Principles and Specific Applications*. Oxford: Blackwell Scientific Publications.

SANNI, M. O. (1992) Gari extract agar—a new medium for mycological research. *Nigerian Journal of Technical Education*, **9**: 1–5.