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Kelulut (Stingless bee) honey - Specification

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Contents

Committee representation

The Industry Standards Committee on Food, Food Products and Food Safety (ISC U) under whose authority this Malaysian Standard was developed, comprises representatives from the following organisations:

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Department of Chemistry, Malaysia
Department of Standards Malaysia
Department of Veterinary Services
Federal Agricultural Marketing Authority
Federation of Malaysian Manufacturers
Jabatan Kemajuan Islam Malaysia
Malaysian Agricultural Research and Development Institute
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The Working Group on *Kelulut* Honey which developed this Malaysian Standard consists of representatives from the following organisations:

Department of Agriculture Malaysia
Department of Standards Malaysia
Kelulut Desa Perak
Malaysia Genome Institute
Malaysian Agricultural Research and Development Institute
Ministry of Agriculture and Agro-Based Industry
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Foreword

This Malaysian Standard was developed by the Working Group on *Kelulut* Honey under the authority of the Industry Standards Committee on Food, Food Products and Food Safety.

Compliance with a Malaysian Standard does not of itself confer immunity from legal obligations.

Kelulut (Stingless bee) honey - Specification

1 Scope

This Malaysian Standard specifies the quality requirements, sampling, preparation of test sample, test methods, hygiene, packaging and labelling for *kelulut* honey produced by stingless bee of Meliponini tribe intended for direct human consumption.

It is applicable to both raw and processed *kelulut* honey.

2 Normative references

The following normative references are indispensable for the application of this standard. For dated references, only the edition cited applies. For undated references, the latest edition of the normative reference (including any amendments) applies.

MS 1514, *General principles of food hygiene*

MS 2679, *Amalan Pertanian Baik (APB) - Pemeliharaan lebah (tribus Apini) dan kelulut (tribus Meliponini)*

Food Act 1983

Food Regulations 1985

Food Hygiene Regulations 2009

3 Terms and definitions

For the purposes of this standard the following terms and definitions apply.

3.1 *kelulut* (stingless bee) honey

A natural sweet with certain acidity substance produced by stingless bees of Meliponini tribe from the nectar of plants or from secretions of living parts of plants, which the stingless bees collect, transform by combining with the specific substances of their own, deposit, dehydrate, store and leave in the natural honey pots to ripen and mature.

In this standard it is referred to as *kelulut* honey.

3.2 processed *kelulut* honey

Raw *kelulut* honey which undergoes drying process at a temperature not more than 40 °C to reduce moisture content.

3.3 raw *kelulut* honey

Kelulut honey that is collected from natural sealed honey pots.

4 Requirements

4.1 General

4.1.1 *Kelulut* honey shall has its natural characteristic flavour and aroma.

4.1.2 *Kelulut* honey shall free from foreign matter.

4.1.3 *Kelulut* honey shall not contain any food additives.

4.1.4 Both raw and processed *kelulut* honey should be harvested and processed in accordance with MS 2679.

4.2 Quality requirements

4.2.1 *Kelulut* honey shall comply with the requirements given in Table 1.

Table 1. Quality requirements for *kelulut* honey

Characteristics	Requirements		Test method[a]
	Raw honey	Processed honey	
Moisture, %	Not more than 35.0	Not more than 22.0	Annex A
Sucrose, g/100 g	Not more than 7.5	Not more than 8.0	Annex B
Fructose and glucose (sum), g/100 g	Not more than 85.0	Not more than 90.0	
Maltose, g/100 g	Not more than 9.5	Not more than 10.0	
Ash, g/100 g	Not more than 1.0	Not more than 1.0	Annex C
Hydroxymethylfurfural, mg/kg	Not more than 30.0	Not more than 30.0	Annex D
pH	2.5 to 3.8	2.5 to 3.8	Annex E
Plant phenolics[b]	Present	Present	Annex F

[a] Other equivalent and recognised test methods can be used.

[b] *Kelulut* honey shall contain naturally occurring plant phenolic. A typical HPLC pattern of plant phenolics in *kelulut* honey is shown in Figure F.1. of Annex F.

4.3 Microbial contaminant limits

Kelulut honey shall not contain microorganisms exceeding the limits specified in Table 2.

Table 2. Microbial contaminant limits for *kelulut* honey

Characteristics	Limit	Test method[a]
Total plate counts, CFU/ml	1×10^3	Annex G
Yeast and mold, CFU/ml	Less than 1×10^1	Annex H
Coliforms, CFU/ml	Less than 1×10^1	Annex J
[a] Other equivalent and recognised test methods can be used. CFU= colony forming unit		

5 Sampling

5.1 General

5.1.1 Different sampling methods are employed for raw *kelulut* honey (5.2) and processed *kelulut* honey (5.3).

5.1.2 The analysis of physicochemical parameters shall be carried out within two to four weeks after collection of samples.

NOTE. Prescribed period indicates the freshness level of the samples for analysis.

5.2 Raw *kelulut* honey

5.2.1 Sampling shall be carried out as the following.

a) Species of the *kelulut* honey shall be identified.

b) The *kelulut* honey shall be harvested from matured honey pots i.e. sealed honey pots by piercing the upper part of the pots using sharp tool followed by suction using clean syringe or specific honey pump.

c) The *kelulut* honey harvested shall be representative of the identified beekeeping area where the sample is taken.

d) The minimum amount of samples to be collected is 350 g.

e) The *kelulut* honey shall be placed in clean, dry and sealed suitable food grade container. It shall be separated according to the specific species.

f) The *kelulut* honey collected shall be kept in a chiller at the temperature between 0 °C to 4 °C.

5.2.2 The samples shall be labelled to include the following information:

a) name of contributors, organisation/company together with contact person and phone number;

b) *kelulut* (stingless bee) species;

c) date of sampling;

d) beekeeping area; and

e) major plant species surrounding the beekeeping area.

5.3 Processed *kelulut* honey

5.3.1 Sampling shall be carried out as the following.

a) Species of the *kelulut* honey and producer of the honey shall be identified.

b) The minimum amount of samples to be collected is 350 g.

c) The *kelulut* honey shall be placed in clean, dry and sealed suitable food grade container. It shall be separated according to the specific species and producer.

5.3.2 The samples shall be labelled to include the following information:

a) name and address of company/manufacturer; and

b) a statement to indicate the samples are processed *kelulut* honey.

6 Preparation of test sample

Crystallised honey should be left at room temperature to allow the crystal to dissolve. Alternatively, the crystallised honey should be heated up to less than 40 °C. Homogenise the sample at room temperature.

7 Test methods

Testing should be carried out as specified in Annexes A to J.

NOTE. Alphabet “I” is not used for labelling of annexes.

8 Packaging and labelling

8.1 Packaging

The product shall be packed in suitable, hygienic, food grade packaging materials which are able to withstand the acidity of *kelulut* honey so as to protect the safety and quality of the product in accordance with MS 2679.

8.2 Labelling

8.2.1 The product shall be labelled in accordance with *Food Act 1983* and *Food Regulations 1985*.

8.2.2 Each container/bottle of product shall be legibly and indelibly labelled with the following information:

a) name of product;

b) batch or code number;

c) date of packing and expiry date;

d) name, and address of the packer;

e) net weight;

f) country of origin;

g) storage instruction; and

h) packer registered trade mark, if any.

8.2.3 The containers may also be marked with the producer specified branding, place of origin and associated plant source.

9 Hygiene

Kelulut honey shall be processed under the good processing and harvesting of *kelulut* honey in accordance with MS 2679, MS 1514 and *Food Hygiene Regulations 2009*.

10 Legal requirements

The product shall in all other aspects comply with the requirements of the legislation currently in force in the country.

Annex A
(normative)

Determination of moisture content by refractometric method

A.1 Scope

This annex describes the procedure to measure the water content of *kelulut* honey.

A.2 Principle

The method is based on the principle that refractive index increases with solids content. The table was constructed from a plot of the logarithm of the refractive index minus unity plotted against the water content as determined by vacuum drying, a technique which requires much greater manipulative skill.

A.3 Apparatus

Use the usual laboratory apparatus and, in particular, the following.

A.3.1 Flasks, 50 ml.

A.3.2 Water bath.

A.3.3 Reichert refractometer or equivalent refractometer, regularly calibrated with distilled water or with another certified reference material. The refractive index for water (n_D) at 20 °C is 1.333 0.

A.4 Procedure

A.4.1 Sample preparation

See Clause 6.

A.4.2 Dissolution

Homogenise the prepared sample again and put in a flask. Close the flask and place in a water bath at 50 °C (± 0.2 °C) until all the sugar crystals are dissolved. Cool the solution to room temperature and stir again. Ensure that the flask is air tight.

A.4.3 Determination

Ensure that the prism of the refractometer is clean and dry. Directly after homogenisation, cover the surface of the prism evenly with the sample. After 2 min, read the refractive index by using the refractometer. Measure each honey thrice and take the average value. Read the corresponding moisture content from Table A.1. Carefully clean the prism after use.

NOTE. The method refers only to the use of the Reichert refractometer, not to digital instruments.

Table A.1. Relationship of water content of *kelulut* honey to refractive index

Water content (g/100 g)	Refractive index at 20 °C	Water content (g/100 g)	Refractive index at 20 °C	Water content (g/100 g)	Refractive index at 20 °C
13.0	1.504 4	17.2	1.493 5	21.4	1.483 0
13.2	1.503 8	17.4	0.493 0	21.6	1.482 5
13.4	1.503 3	17.6	1.492 5	21.8	1.482 0
13.6	1.502 8	17.8	1.492 0	22.0	1.481 5
13.8	1.502 3	18.0	1.491 5	22.2	1.481 0
14.0	1.501 8	18.2	1.491 0	22.4	1.480 5
14.2	1.501 2	18.4	1.490 5	22.6	1.480 0
14.4	1.500 7	18.6	1.490 0	22.8	1.479 5
14.6	1.500 2	18.8	1.489 5	23.0	1.479 0
14.8	1.499 7	19.0	1.489 0	23.2	1.478 5
15.0	1.499 2	19.2	1.488 5	23.4	1.478 0
15.2	1.498 7	19.4	1.488 0	23.6	1.477 5
15.4	1.498 2	19.6	1.487 5	23.8	1.477 0
15.6	1.497 6	19.8	1.487 0	24.0	1.476 5
15.8	1.497 1	20.0	1.486 5	24.2	1.476 0
16.0	1.496 6	20.2	1.486 0	24.4	1.475 5
16.2	1.496 1	20.4	1.485 5	24.6	1.475 0
16.4	1.495 6	20.6	1.485 0	24.8	1.474 5
16.6	1.495 1	20.8	1.484 5	25.0	1.474 0
16.8	1.494 6	21.0	1.484 0		
17.0	1.494 0	21.2	1.483 5		

NOTES:

1. Value for 20 °C are Wedmore's calculation, Bee World 36. 197-206 (1955).
2. If refractive index is measured at temperatures above 20 °C, add 0.000 23 per degree and if measured below 20 °C, substract 0.000 23 per degree before using table.

The table is derived from a formula developed by Wedmore from the data of Chataway and others as the following equation:

$$W = \frac{1.731\ \ 90 - \log\left(R.I - 1\right)}{0.002\ 243}$$

where

W is the water content in g per 100 g honey; and

$R.I$ is the refractive index.

Annex B
(normative)

Determination of sugar content

B.1 Scope

This annex describes the procedure to determine fructose, glucose, sucrose and maltose, in *kelulut* honey, for which precision data are acquired.

B.2 Principle

After filtration of the solution, the sugar content is determined by HPLC (High Performance Liquid Chromatography) with refractive index (R.I) detection. Peaks are identified on the basis of their retention times. Quantification is performed according to the external standard method on peak areas or peak heights.

B.3 Reagents

Unless otherwise specified, chemicals shall be of recognised analytical reagent quality and distilled water should be used in the test.

B.3.1 **Methanol**, HPLC grade.

B.3.2 **Acetonitrile**, HPLC grade.

B.3.3 **Eluent solution for the HPLC**, mix 80 volumes of acetonitrile with 20 volumes of water. Degas prior to use.

B.3.4 **HPLC water or of equivalent purity**, for example double distilled deionised water.

B.3.5 **The standard solution**, fructose, glucose, sucrose and maltose.

Pipette 25 ml methanol into a 100 ml volumetric flask. Depending on the sugars to be analysed, dissolve the amount of each standard substance as in Table B.1., approximately in 40 ml water and transfer quantitatively to the flask and fill to the mark with water.

Table B.1. Amount of standard substances for the various types of sugars

Type of sugar	Amount of sugar (g)
Fructose	2.000
Glucose	1.500
Sucrose	0.250
Maltose	0.150

Use a syringe and pre-mounted membrane filter to transfer the solution to sample vials. The standard solutions are stable for four weeks in the refrigerator at 4 °C and for six months at 18 °C.

B.4 Apparatus

Use the usual laboratory apparatus and, in particular, the following.

B.4.1 Vials.

B.4.2 Ultrasonic bath.

B.4.3 Volumetric flask, 100 ml.

B.4.4 Pipette, 2 ml.

B.4.5 Membrane filter for aqueous solutions, pore size 0.45 μm.

B.4.6 Filter holder for membrane filters with suitable syringe.

B.4.7 HPLC, consisting of pump, sample applicator, temperature-regulated RI-detector thermostated at 30 °C, temperature regulated column oven at 30 °C and integrator.

B.4.8 Analytical stainless-steel column, 4.6 mm in diameter, 250 mm length, containing amine-modified silica gel with 5 μm to 7 μm particle size. Before using, carry out a system suitability test to ensure all the sugars can be separated.

B.4.9 Analytical balance, accurate to 0.001 g.

B.5 Procedure

B.5.1 Sample preparation

See Clause 6.

B.5.2 Preparation of the sample solution

Weigh 5 g of honey into a beaker and dissolve in 40 ml water. Pipette 25 ml of methanol into a 100 ml volumetric flask and transfer the honey solution quantitatively to the flask. Fill to the mark with water. Filter through a membrane filter and collect in sample vials. Store as that of the standard solution (B.3.5).

B.5.3 High Performance Liquid Chromatography (HPLC) condition

If a column of the type described above is used, the following conditions are recommended:

Flow rate: 1.3 ml/min.

Mobile phase: Acetonitrile:water (80:20, *v/v*).

Column and detector temperature: 30 °C.

Sample volume: 10 μl.

NOTES.

1. If it is not possible to carry out analysis at 30 °C and if the detector cannot be thermostated at 30 °C, carry out the analysis at ambient temperature.

2. Identical volumes of sample and standard solution should be injected.

B.6 Calculation and expression of results

B.6.1 The honey sugars are identified and quantified by comparison of the retention times and the peak area of the honey sugars with those of the standard sugars.

B.6.2 The weight percentage of the sugars to be determined of fructose, glucose, maltose and sucrose in g/100 g is calculated according to the following formula:

$$\frac{A_1 \times V_1 \times m_1}{A_2 \times V_2 \times m_0}$$

where

A_1 is the peak area or peak heights of the given sugar compound in the sample solution,expressed as units of area, length or integration;

A_2 is the peak heights of the given sugar compound in the standard solution, expressed as units of area, length or integration;

V_1 is the total volume of the sample solution in ml;

V_2 is the total volume of the standard solution in ml;

m_1 is the weight of the sugar in grammes in the total volume of standard (V_2); and

m_0 is the sample weight in grammes.

Round the result to one decimal place.

Annex C
(normative)

Determination of ash content

C.1 Scope

This annex prescribes the procedure to determine the ash content in *kelulut* honey.

C.2 Principle

The honey is ashed at a temperature of less than 600 °C and the residue is weighed out.

C.3 Reagents

Unless otherwise specified, chemicals shall be of recognised analytical reagent quality and distilled water should be used in the test.

C.3.1 Olive oil, free from ash.

C.4 Apparatus

Use usual laboratory apparatus and, in particular, the following.

C.4.1 Platinum or quartz or porcelain ash dish, of suitable size.

C.4.2 Appliance for preliminary ashing, such as an infra-red heater, a gas burner or a hot plate.

C.4.3 Electric furnace, adjustable to 600 °C (± 25 °C).

C.4.4 Desiccator, containing an efficient desiccant.

C.4.5 Analytical balance, accurate to 0.1 mg.

C.5 Procedure

C.5.1 Preparation of the ash dish

Dry the ash dish in the electrical furnace at ashing temperature, subsequently cool in a desiccator to room temperature and weigh to 0.001 g.

C.5.2 Sample preparation

See Clause 6.

C.5.3 Determination

C.5.3.1 Weigh 5 g to 10 g of the sample to the nearest 0.001 g into the dried ash dish. Add two drops of olive oil. Commence preliminary ashing without loss at a low heat rising to 350 °C to 400 °C by using one of the appliances, until the sample is black and there is no loss by foaming.

C.5.3.2 After the preliminary ashing, place the dish in the preheated furnace and heat for at least 1 h. Cool the ash dish in the desiccator and weigh. Continue the ashing procedure until constant weight is reached.

C.6 Calculation and expression of results

The ash content in honey, expressed as percentage by weight is calculated using the following formula:

$$\frac{m_1 - m_2}{m_0} \times 100$$

where

m_0 is the weight of honey taken;

m_1 is the weight of dish and ash; and

m_2 is the weight of dish.

Round the result to two decimal places.

Annex D
(normative)

Determination of hydroxymethylfurfural by spectrophotometric method

D.1 Scope

This annex describes the procedure to determine hydroxymethylfurfural in *kelulut* honey using spectrophotometric method.

D.2 Principle

The determination of hydroxymethylfurfural (HMF) content is based on the determination of UV absorbance of HMF at 284 nm. In order to avoid the interference of other components at this wavelength the difference between the absorbances of a clear aqueous honey solution and the same solution after addition of bisulphite is determined. The HMF content is calculated after subtraction of the background absorbance at 336 nm. This method is based on the original work of White.

D.3 Reagents

Unless otherwise specified, chemicals shall be of recognised analytical reagent quality and distilled water should be used in the test.

D.3.1 Carrez solution I, dissolve 15 g $K_4Fe(CN)_6 \cdot 3H_2O$ and dilute to 100 ml with H_2O.

D.3.2 Carrez solution II, dissolve 30 g $Zn(CH_3COO)_2 \cdot 2H_2O$ and dilute to 100 ml with H_2O.

D.3.3 Sodium bisulphite solution, 0.20 %, dissolve 0.20 g $NaHSO_3$ (technical grade is satisfactory) and dilute to 100 ml with H_2O. Dilute 1 + 1 for dilution of reference solution if necessary. Prepare fresh daily.

D.4 Apparatus

Use usual laboratory apparatus and, in particular, the following.

D.4.1 Spectrophotometer UV, to measure A at 284 nm and 336 nm.

D.5 Procedure

D.5.1 Sample preparation

See Clause 6.

D.5.2 Determination

D.5.2.1 Accurately weigh 5 g honey in small beaker and transfer with total of 25 ml H_2O to 50 ml volumetric flask. Add 0.50 ml Carrez solution I, mix, add 0.50 ml Carrez solution II, mix, and dilute to volume with H_2O. Drop of alcohol may be added to suppress foam. Filter through paper, discarding first 10 ml filtrate.

D.5.2.2 Pipet 5 ml filtrate into each of two 18 mm x 150 mm test tubes. Add 5.0 ml H_2O to one tube (sample) and 5.0 ml $NaHSO_3$ solution to other (reference). Mix well.

D.5.2.3 Determine A of sample against reference at 284 nm and 336 nm in 1 cm cells. If A is less than 0.6, dilute sample solution with H_2O and reference solution with 0.1 % $NaHSO_3$ solution to same extent and correct A for dilution.

D.6 Calculation and expression of results

The HMF content, expressed in mg/kg, of the sample is calculated using the following formula:

$$\frac{(A_{284} - A_{336}) \times 149.7 \times 5 \times D}{W}$$

where

A_{284} is the absorbance at 284 nm;

A_{336} is the absorbance at 336 nm;

149.7 is the factor = $\frac{126 \times 1\,000 \times 1\,000}{16\,830 \times 10 \times 5}$;

126 is the molecular weight of HMF;

16 830 is the molar absorptivity of HMF, λ at 284 nm;

1 000 is the conversion g into mg;

10 is the conversion 5 into 50 ml;

1 000 is the conversion g of honey into kg;

5 is the theoretical nominal sample weight;

D is the dilution factor, in case dilution is necessary; and

W is the weight of honey taken.

Results are expressed in mg/kg to one decimal place.

Annex E
(normative)

Determination of pH

E.1 Scope

This annex describes the procedure to determine the pH of *kelulut* honey.

E.2 Principle

The sample is dissolved in water and the pH is measured.

E.3 Reagents

Unless otherwise specified, chemicals shall be of recognised analytical reagent quality and distilled water should be used in the test.

E.3.1 Distilled water, carbon dioxide-free.

E.3.2 Buffer solutions, for calibration of the pH meter at pH 3.7 (or pH 4.0), pH 7.0 and pH 9.0.

E.4 Apparatus

Use the usual laboratory apparatus and, in particular, the following.

E.4.1 pH meter, accurate to 0.01 units.

E.4.2 Magnetic stirrer.

E.4.3 Beaker, 250 ml.

E.5 Procedure

E.5.1 Calibration of pH meter

E.5.1.1 The meter should be calibrated at pH 7 (or pH 4.0), pH 7.0 and pH 9.0.

E.5.2 Sample preparation

See Clause 6.

E.5.3 Determination

Dissolve 10 g sample in 75 ml of carbon dioxide-free water in a 250 ml beaker. Stir with the magnetic stirrer, immerse the pH electrodes in the solution and record the pH.

E.6 Expression of results

Report the pH to two decimal places.

Annex F
(normative)

Determination of plant phenolics

F.1 Scope

This annex describes the procedure to determine the plant phenolics in *kelulut* honey.

F.2 Principle

Plant phenolics contents are determined by HPLC (High Performance Liquid Chromatography) with photo diode array detector (PDA) and/or with mass spectrometry detector. Peaks are identified on the basis of their retention times, UV spectra and reference standards. Quantifications are performed according to the external standard method on peak areas.

F.3 Reagents

Unless otherwise specified, chemicals shall be of recognised analytical reagent quality and distilled water should be used in the test.

F.3.1 **Methanol**, HPLC grade.

F.3.2 **Acetonitrile**, HPLC grade.

F.3.3 **Eluent solution for the HPLC**, gradient mixing of acetonitrile and water containing 1.0 % formic acid. The gradient elution is carried out over 70 min from 10 % to 30 % acetonitrile.

F.3.4 **HPLC water or of equivalent purity**, for example double distilled deionised water.

F.4 Apparatus

Use the usual laboratory apparatus and, in particular, the following.

F.4.1 **Membrane filter** for aqueous solutions, pore size 0.45 μm.

F.4.2 **HPLC** consisting of pump, sample applicator, temperature-regulated PDA-detector thermostated at 30 °C, temperature regulated column oven at 30 °C and integrator.

F.4.3 **Analytical stainless-steel column**, e.g. 5 μm, 4.6 mm x 250 mm.

F.4.4 **Analytical balance**, accurate to 0.001 g.

F.5 Procedure

F.5.1 Sample preparation

See Clause 6.

F.5.2 High Performance Liquid Chromatography (HPLC) condition

If a column of the type described above is used, the following conditions are recommended:

Flow rate: 0.2 ml/min.

Mobile phase: Acetonitrile:water containing 1.0 % formic acid. The gradient elution is carried out over 70 min from 10 % to 30 % acetonitrile

Column and detector temperature: 40 C.

Sample volume: 10 µl.

F.5.3 Determination

F.5.3.1 Acidify 4.0 g sample to pH 2.0 with 10 ml hydrochloric acid (HCl).

F.5.3.2 Use solid phase extraction (SPE) C18 column cartridge for sample purification.

F.5.3.3 Pre-condition the cartridge with methanol and HCl (pH 2) and load the loading with the acidified sample.

F.5.3.4 After adding the acidified sample, wash the cartridge with methanol. The extract is then reduced to dryness *in vacuo*.

F.5.3.5 Analyse extract of sample in triplicate using HPLC comprising HPLC pump with an auto sampler and a photo diode array detector (PDA) scanning from 200 nm to 550 nm. Plant phenolics can be detected at 280 nm and 365 nm.

F.6 Expression of results

A typical of HPLC pattern of plant phenolics in *kelulut* honey is shown in Figure F.1.

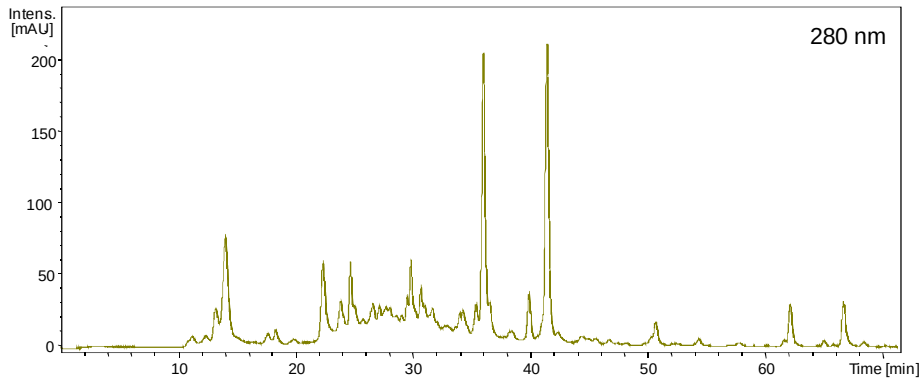

Figure F.1. HPLC chromatogram of plant phenolics in *kelulut* honey

Annex G
(normative)

Determination of total plate count

G.1 Scope

This annex describes the procedure to determine the total number of aerobic bacterial population on *kelulut* honey.

G.2 Principle

The total plate count is intended to indicate the level of microorganism in *kelulut* honey. The suitable colony counting range is 25 colonies to 250 colonies. The plate count method means diluting bacteria with a diluent solution (e.g. sterile saline) until the bacteria are diluted enough to count accurately when spread on a plate. The assumption is that each viable bacterial cell will develop into a single colony. Bacterial cell numbers need to be reduced by dilution, because more than 200 colonies on a standard 9 cm plate are likely to produce colonies too close to each other to be distinguished as distinct colony-forming units (CFUs).

G.3 Reagents

Unless otherwise specified, chemicals shall be of recognised analytical reagent quality and distilled water should be used in the test.

G.3.1 **Sterile Ringers solution**, for dilution.

G.3.2 **Sterile plate count agar**.

G.4 Apparatus

Use the usual laboratory apparatus and, in particular, the following.

G.4.1 **Sterile petri dishes**, glass or plastic, at least 15 mm × 90 mm.

G.4.2 **Pipets and sterile pipet tips**, 1 ml.

G.4.3 **Dilution bottles**, 100 ml and 25 ml.

G.4.4 **Circulating water bath**, thermostatically controlled to 45°C ± 1°C.

G.4.5 **Incubator**, 37°C ± 1°C.

G.4.6 **Colony counter**.

G.5 Procedure

G.5.1 Sample preparation

See Clause 6.

G.5.2 Determination

G.5.2.1 Weigh 10 g sample into 90 ml Ringers solution and shake for 25 times within 7 s.

G.5.2.2 Using separate sterile pipets, prepare decimal dilutions of 10^{-2}, 10^{-3}, 10^{-4}, and others as appropriate by transferring 1.0 ml of previous dilution to 9.0 ml of diluents.

G.5.2.3 Pipet 1 ml of each dilution into separate, duplicate, appropriately marked petri dishes.

G.5.2.4 Add 12 ml to15 ml plate count agar (cooled to 45°C ± 1°C) to each plate within 15 min of original dilution.

G.5.2.5 Immediately mix sample dilutions and agar medium thoroughly and uniformly by alternate rotation and back-and-forth motion of plates on flat level surface. Let agar solidify.

G.5.2.6 Invert solidified petri dishes, and incubate promptly for 48 h ± 2 h at 37 °C.

G.5.3 Counting of colonies

G.5.3.1 After incubation period, count growth colonies in all petri dish.

G.5.3.2 Normal plates (25 colonies to 250 colonies): Select spreader-free plate(s). Count all colony forming units (CFU), including those of pinpoint size, on selected plate(s). Record dilution(s) used and total number of colonies counted.

G.5.3.3 Plates with more than 250 colonies: When number of CFU per plate exceeds 250 colonies, for all dilutions, record the counts as too numerous to count (TNTC).

G.5.3.4 Plates with no CFU: When plates from all dilutions have no colonies, report APC as less than 1×10^1 the corresponding lowest dilution used.

Annex H
(normative)

Enumeration of yeast and mold

H.1 Scope

This annex prescribes the procedure to enumerate the yeast and mold on *kelulut* honey.

H.2 Principle

There are many media for enumeration of yeast and mold in food samples. These media are either acidified or incorporated with antibiotic to suppress the growth of bacteria. In general, fungi can grow at an optimum pH much lower than that most of bacteria. Selective medium acidified to around pH 3.5 using sterile lactic or tartaric acid has been traditionally used for enumerating yeast and mold. Acidified media presents two distinct shortcoming- occasional growth of acidic tolerant bacteria and some yeast and mold grow poorly. Although acidified medium is preferred when examining acidic sample, it is not suitable for food media containing high level of acid tolerant bacteria.

H.3 Reagents

Unless otherwise specified, chemicals shall be of recognised analytical reagent quality and distilled water should be used in test.

H.3.1 **Sterile Ringers solution**, for dilution.

H.3.2 **Sterile Malt Extract Agar (MEA)**.

H.3.3 **Tartaric acid**, 10 %.

H.4 Apparatus

Use the usual laboratory apparatus and, in particular, the following.

H.4.1 **Sterile petri dishes**, glass or plastic, at least 15 mm × 90 mm.

H.4.2 **Pipets and sterile pipet tips**, 1 ml.

H.4.3 **Dilution bottles**, 100 ml and 25 ml.

H.4.4 **Circulating water bath**, thermostatically controlled to 45 C ± 1 C.

H.4.5 **Incubator**, 37°C ± 1 C.

H.4.6 **Colony counter**.

H.5 Procedure

H.5.1 Sample preparation

See Clause 6.

H.5.2 Determination

H.5.2.1 Weigh 10 g sample into 90 ml Ringers solution and shake for 25 times within 7 s.

H.5.2.2 Using separate sterile pipets, prepare decimal dilutions of 10^{-2}, 10^{-3}, 10^{-4}, and others as appropriate by transferring 1.0 ml of previous dilution to 9.0 ml of diluents.

H.5.2.3 Pipet 1 ml of each dilution into separate, duplicate, appropriately marked petri dishes.

H.5.2.4 Add 12 ml to 15 ml MEA (added with 1.2 ml tartaric acid 10 % to 100 ml agar) (cooled to 45 °C ± 1 °C) to each plate within 15 min of original dilution.

H.5.2.5 Immediately mix sample dilutions and agar medium thoroughly and uniformly by alternate rotation and back-and-forth motion of plates on flat level surface. Let agar solidify.

H.5.2.6 Invert solidified petri dishes, and incubate promptly for 72 h ± 2 h at 25 °C.

H.5.3 Counting of colonies

H.5.3.1 After incubation period, count grown colonies in all petri dish.

H.5.3.2 Normal plates (25 colonies to 250 colonies): Select spreader-free plate(s). Count all colony forming units (CFU), including those of pinpoint size on selected plate(s). Record dilution(s) used and total number of colonies counted.

H.5.3.3 Plates with more than 250 colonies: When number of CFU per plate exceeds 250 colonies, for all dilutions, record the counts as too numerous to count (TNTC).

H.5.3.4 Plates with no CFU: When plates from all dilutions have no colonies, report APC as less than 1×10^1 the corresponding lowest dilution used.

Annex J
(normative)

Determination of total coliforms

J.1 Scope

This annex prescribes the procedure to determine the coliforms count on *kelulut* honey.

J.2 Principle

The coliform group includes aerobic and facultatively anearobic, gram negative, non-sporing rods that ferment lactose, with acid and gas production within 48 h at temperatures between 30 °C and 37 °C. The bacteria detected by coliform test are members of several genera within the family Enterobacteriacae including *Escherichia*, *Enterobacter*, *Klebsiella* and *Citrobacter*. The sample is inoculated into the medium containing lactose, which is normally made selective by addition of bile salts, other surface active agents of dyes.

Viable Red Bile Agar (VRBA) is a lactose containing selective medium for the detection and enumeration of coliforms or lactose fermenting gram negative bacteria. The medium contains bile salt and crystal violet as selective agents and the lactose fermentation is indicated by the pH indicator dye, neutral red. The medium pasteurised by boiling. Organisms which rapidly attack lactose produce purple (dark) colonies surrounded by purple haloes (reddish zone of precipitated bile). Non-lactose or late-lactose fermenters produce pale or colourless colonies with greenish zones. Coliforms on this medium produce dark red colonies usually greater than 0.5 mm in diameter and often surrounded by a reddish zone. All colonies are in the depth of the agar due to the overlay.

J.3 Reagents

Unless otherwise specified, chemicals shall be of recognised analytical reagent quality and distilled water should be used in test.

J.3.1 **Sterile Ringers solution**, for dilution.

J.3.2 **Sterile Viable Red Bile Agar (VRBA)**.

J.4 Apparatus

Use the usual laboratory apparatus and, in particular, the following:

J.4.1 **Sterile petri dishes**, glass or plastic, at least 15 mm × 90 mm.

J.4.2 **Pipets and sterile pipet tips**, 1 ml.

J.4.3 **Dilution bottles**, 100 ml and 25 ml.

J.4.4 **Circulating water bath**, thermostatically controlled to 45°C ± 1 C.

J.4.5 **Incubator**, 37°C ± 1 C.

J.4.6 **Colony counter**.

J.5 Procedure

J.5.1 Sample preparation

See Clause 6.

J.5.2 Determination

J.5.2.1 Weigh 10 g sample into 90 ml Ringers solution and shake for 25 times within 7 s.

J.5.2.2 Using separate sterile pipets, prepare decimal dilutions of 10^{-2}, 10^{-3}, 10^{-4}, and others as appropriate by transferring 1.0 ml of previous dilution to 9.0 ml of diluents.

J.5.2.3 Pipet 1 ml of each dilution into separate, duplicate, appropriately marked petri dishes.

J.5.2.4 Add 10 ml VRBA (cooled to 45°C ± 1°C) to each plate within 15 min of original dilution.

J.5.2.5 Immediately mix sample dilutions and agar medium thoroughly and uniformly by alternate rotation and back-and-forth motion of plates on flat level surface. Let agar solidify.

J.5.2.6 Add another layer of agar onto previous solidified agar. Let agar solidify.

J.5.2.7 Invert solidified petri dishes, and incubate promptly for 48 h ± 2 h at 37 °C.

J.5.3 Counting of colonies

J.5.3.1 After incubation period, count grown colonies in all petri dish.

J.5.3.2 Normal plates (25 colonies to 250 colonies): Select spreader-free plate(s). Count all colony forming units (CFU), including those of pinpoint size on selected plate(s). Record dilution(s) used and total number of colonies counted.

J.5.3.3 Plates with more than 250 colonies: When number of CFU per plate exceeds 250 colonies, for all dilutions, record the counts as too numerous to count (TNTC).

J.5.3.4 Plates with no CFU: When plates from all dilutions have no colonies, report APC as less than 1 x 10^1 the corresponding lowest dilution used.

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