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Avocado Honey — Specification

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Foreword

This Ethiopian Standard has been prepared under the direction of the Technical Committee for spice and condiments (TC 14) and published by the Institute of Ethiopian Standards (IES).

The standard has been developed to address observed needs and to support the local industry in order to make progress through uprising competitiveness and maintain comparative market advantage both domestically and internationally.

In preparing this standard reference has been made to the following documents:

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Avocado Honey — Specification

1. Scope

This Ethiopian Standard specifies requirements for avocado honey produced by *Apis mellifera* bees. Avocado honey from other bee species may also be included, provided it satisfies the criteria outlined in this standard and is intended for human consumption.

The standard also covers avocado honey which is packed in non-retail (bulk) containers and is intended for re-packing into retail packs.

Avocado honey that is not intended for human consumption as food is not covered by this Standard.

2. Normative References

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

CES 73 – General standard for pre-packaged foods labeling

ES 361 – Canned fruits and vegetables, heat preserved, Determination of Tin content

ES ISO 6634 – Fruits, vegetables, and derived products – Determination of arsenic content – Silver diethyldithiocarbamate spectrophotometric method

ES ISO 17043- General requirements for proficiency testing-Applied to inter-laboratory comparisons in honey pollen testing

3. Terms and Definitions

For the purpose of this standard, the following definitions shall apply.

3.1
honey
the natural sweet substance produced by honey bees from the nectar of plants or from secretions of living parts of plants or excretions of plant-sucking insects on the living parts of plants, which the bees collect, transform by combining with specific substances of their own, deposit, dehydrate, store, and leave in the honeycomb to ripen and mature.

3.2
avocado honey
a mono-floral honey obtained exclusively from the blossoms (nectar) of the avocado tree (*Persea americana*).

3.3
blossom honey or nectar honey
is the honey which is derived mainly from the nectar of flowers.

3.4
extracted honey
honey only obtained by centrifuging decamped broodless combs.

3.5
pressed honey
honey obtained by pressing broodless combs with or without the application of moderate heat.

3.6
comb honey
honey stored by honey bees in the cells of freshly built broodless combs or thin comb foundation sheets made solely of beeswax and sold in sealed whole combs or sections of such combs.

3.7
chunk honey
chunks of cut comb honey suspended in a medium of extracted honey a dark amber to reddish-brown color.

3.8
perseitol
a unique sugar present only in avocado honey, served as an indicator for the degree of purity of avocado honey.

4. Requirement

4.1. General Requirement

4.1.1. All sugars including perseitol in honey shall have been collected by honey bees from the plants' nectar and honeydew and living parts of plants.

4.1.2. No specific constituent (which characterizes the botanical and geographical origin or bioactivity) shall be added nor removed from the honey.

4.1.3. The honey shall not show signs of yeast fermentation or effervescence.

4.1.4. Honey shall have its natural characteristic color, taste, aroma, viscosity and consistent appearance.

4.1.5. The color shall vary from dark amber to reddish-brown color.

4.1.6. Honey shall possess the natural taste, aroma, and color typical of avocado.

4.1.7. Honey shall be free from toxins which may constitute a health hazard.

4.1.8. When visually inspected, the honey shall be free from foreign matter such as bees, dirt, scum, beeswax or any other extraneous matter.

Note: Any techniques that use a significant reduction in air pressure (vacuum air) to reduce the water content of the honey is not permitted.

4.2. Specific requirements

Table 1 Specific Requirements for Avocado honey

Characteristic		Requirements	Test Methods
Relative density (S.G.), Min.		1.37	A
Moisture, % by mass, Max.		20	B
Glucose and Fructose, g/100g, Min.		60	C
Sucrose, % by mass, Max.		5	C, D
Pollen content, %, Min.		25	xxxxxxxx
Colour, mm Pfund, Min.		100	xxxxxxxx
Perseitol, Min.		0.2 if pollen count i 25	xxxxxxxx
Electrical Conductivity, mS/cm, Min.		0.7	E
Free acidity expressed as milli equivalent acid/kg honey, Max.		50	F
Diastase Activity (Determined after processing), Schade Units,		8	G
Hydroxymethyl furfural (HMF), mg/kg, Max.		40	H
Water insoluble solids content, % by mass, Max.	Pressed honey	0.5	I
	Extracted honey	0.1	
Proline content, mg/kg, Min		180	J

5. Food additives

5.1. Avocado honey shall not contain any added substances, either in the form of additions or additives.

5.2. Avocado honey acidity shall not be changed artificially.

6. Contaminants

6.1. Metal contaminants

6.1.1. Avocado honey shall be free from heavy metals in amounts which may represent a hazard to human health.

6.1.2. The products covered by this Standard shall comply with those maximum levels for heavy metals established by the Codex Alimentarius Commission.

In particular, the levels of metallic contaminants in avocado honey shall not exceed limits as follows.

Table 2 Limits for metal contaminants

Characteristic	Maximum limits, mg/kg (ppm)	Test Methods
Arsenic	0.1	ES ISO 6634
Lead	0.1	AOAC 999.10
Copper	0.1	AOAC 999.10
Zinc	5.0	AOAC 999.10
Tin	150	ES 361

6.2. Residues of pesticides and veterinary drugs

The products covered by this standard shall comply with those maximum residue limits for honey established by the Codex Alimentarius Commission.

7. Hygiene

It is recommended that the products covered by the provisions of this standard be prepared and handled in accordance with the appropriate sections, Hazard analysis critical control points (HACCP).

7.1. The products should comply with any microbiological criteria established in accordance with the *Principles for the Establishment and Application of Microbiological Criteria for Foods* (CAC/GL 21-1997).

7.2. Avocado honey shall be free from visible mold and as far as practicable, be free from inorganic or organic matter foreign to its composition, such as insects, insect debris, brood or grains of sand, or soil, when the honey is offered for sale or is used in any product for human consumption.

7.3. Avocado honey should not contain toxic substances arising from microorganisms or plants in an amount, which may constitute a hazard to health.

8. Packing

8.1. Avocado Honey shall be packed in suitable packages which shall be clean, sound and free from any contaminants. The packaging material shall be of food grade quality and shall be securely closed and sealed.

8.2. Packing materials shall not impart any odor to the honey. Particularly if used containers are recycled, care shall be taken that they are absolutely clean and have no residual odor.

9. Labelling

In addition to the relevant sections of CES 73 the following specific provisions shall also apply:

a) Name of the product **"Avocado honey"**.

b) Net weight in SI unit

c) Name and address of the manufacturer/packer

d) Production year (dd/mm/yy)

e) Lot/Batch or code number

f) Expiry date (dd/mm/yy)

g) Country of origin

h) Geographical indication (GI) for Ethiopian avocado-growing regions - (optional)

10. Sampling Methods

Sampling shall be in accordance with the procedure indicated in Annex K

Annex A
(Normative)
Determination of relative density

A.1Apparatus

A.1.1Thermostatically controlled water bath — Maintainable at 27 ^{0}C±1 ^{0}C.

A.1.2Specific gravity bottle

A.2 Procedure

Clean and thoroughly dry the specific bottle and weigh. Fill it up to the mark with freshly boiled and cooled distilled water which has been maintained at 27 ^{0}C ± 1 ^{0}C and weigh. Remove the water, dry the bottle again and fill it with the honey sample maintained at the same temperature. Weigh the bottle again.

A.3 Calculation

$$\text{Relative density} = \frac{C - A}{B - A}$$

Where

A is mass in g of the empty specific gravity bottle;

B is mass in g of the specific gravity bottle with water; and

C is mass in g of the specific gravity bottle with honey sample.

Annex B
(Normative)
Determination of moisture content

Two methods have been specified a) Refractometer method and b) Oven drying method.Oven drying method should be used as reference method; however, refractometer method may be used for routine analysis of honey with moisture content up to 25 percent.

B.1 Refractometer method

B.1.1 Apparatus —Refractometer

B.1.2 Procedure

B.1.2.1 Sample preparation.

Dissolution

Homogenize the prepared sample again and put in a flask. Close the flask and place in a water bath at 50^0C (± 0.2) until all the sugar crystals are dissolved. Cool the solution to room temperature and stir again.

Note: Ensure that the flask is air tight.

B.1.2.2 Determination

Ensure that the prism of the refractometer is clean and dry. Directly after homogenization, cover the surface of the prism evenly with the sample. After 2 minutes (Abbe refractometer) read the refractive index. Measure each honey twice and take the average value. Read the corresponding moisture content from the table. Carefully clean the prism after use.

Note: The method refers only to the use of the Abbe refractometer, not to digital instruments. Determine the refractometer reading of honey at 20 ^{0}C and calculate the percentage of moisture from the values given in Table B.1. If the determination is made at a temperature other than 20 ^{0}C, correct the reading according to the Note in Table B.1.

Table 1 Refractive indices, corresponding percent soluble solids, and percent moisture in extracted honey

Water Content, g/100 g	Refractive Index 20^0C	Water Content g/100 g	Refractive Index 20^0C
13.0	1.5044	19.0	1.4890
13.2	1.5038	19.2	1.4885
13.4	1.5033	19.4	1.4880
13.6	1.5028	19.6	1.4875
13.8	1.5023	19.8	1.4870
14.0	1.5018	20.0	1.4865
14.2	1.5012	20.2	1.4860
14.4	1.5007	20.4	1.4855
14.6	1.5002	20.6	1.4850
14.8	1.4997	20.8	1.4845
15.0	1.4992	21.0	1.4840
15.2	1.4987	21.2	1.4835
15.4	1.4982	21.4	1.4830
15.6	1.4976	21.6	1.4825
15.8	1.4971	21.8	1.4820
16.0	1.4966	22.0	1.4815
16.2	1.4961	22.2	1.4810

16.4	1.4956	22.4	1.4805
16.6	1.4951	22.6	1.4800
16.8	1.4946	22.8	1.4795
17.0	1.4940	23.0	1.4790
17.2	1.4935	23.2	1.4785
17.4	1.4930	23.4	1.4780
17.6	1.4925	23.6	1.4775
17.8	1.4920	23.8	1.4770
18.0	1.4915	24.0	1.4765
18.2	1.4910	24.2	1.4760
18.4	1.4905	24.4	1.4755
18.6	1.4900	24.6	1.4750
18.8	1.4895	24.8	1.4745
		25.0	1.4740

Temperatures above 20 ^{0}C: add 0.00023 per ^{0}C.

Temperatures below 20 ^{0}C: subtract 0.00023 per ^{0}C.

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

$$W = \frac{1.73190 - log\ (R.I - 1)}{0.002243}$$

Where,

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

R.I is the refractive index

B.2 Oven drying method

B.2.1 Apparatus

B.2.1.1 Flat-bottom dish — of nickel or other suitable material not affected by boiling water; 7 cm to 8 cm in diameter and not more than 2.5 cm deep.

B.2.1.2 Sand Passing through 500 -micron sieve but retained on 180-micron sieve. It shall be prepared by digestion with concentrated hydrochloric acid, followed by thorough washing with water till free from chlorides. It shall be dried and ignited to dull red heat.

B.2.1.3 Vacuum oven

B.2.2 Procedure

B.2.2.1 Heat the dish containing 20 g of the prepared sand and a stirring rod in the oven for one hour. Allow to cool in an efficient desiccator for 30 to 40 minutes. Weigh accurately 2 g of the material into the tared dish. Add 5 ml of distilled water in the dish and thoroughly mix sand with the sample by stirring with the glass rod having a widened flat end, smoothing out lumps and spreading the mixture over the bottom of the dish.

B.2.2.1.1 Place the dish on a boiling water-bath f or 30 minutes. Wipe the bottom of the dish and transfer it, with the glass rod, to the vacuum oven maintained at a temperature between 60°C and 70°C and at a pressure not more than 50mm of mercury.

B.2.2.1.2 After 2 hours, remove the dish and transfer to a desiccator, allow it to cool and then weigh. Replace the dish in the oven for a further period of one hour, remove and transfer to the desiccator, cool and weigh again. Repeat the process of heating, cooling and weighing after every one hour till consecutive weighings do not differ by more than 0.5 mg.

B.2.3 Calculation

$$\textit{Moisture, percent by mass} = \frac{100\,(M1 - M2)}{M1 - M2}$$

where

M_1 = mass, in g, of the contents of the dish before drying;

M_2 = mass, in g, of the contents of dish after drying; and

M = mass, in g, of the empty dish with the sand and the glass rod.

Annex C
(Normative)
Determination of sugars by HPLC

C.1 PRINCIPLE

This method is based on the originally published method by Bogdanov and Baumann (1). After filtration of the solution, the sugar content is determined by HPLC (High Pressure Liquid Chromatography) with RI-detection. Peaks are identified on the basis of their retention times. Quantitation is performed according to the external standard method on peak areas or peak heights.

C.2 REAGENTS

If not stated otherwise, chemicals of analytical purity grade should be used.

The water must be distilled or should be of at least equivalent purity.

Methanol for HPLC

Acetonitrile for HPLC

Warning: Acetonitrile is a dangerous substance. Laboratory safety guidelines on dangerous substances at work should be consulted.

Eluent solution for the HPLC.Mix 80 volumes of acetonitrile with 20 volumes of water.Degass prior to use.

The standard substances, fructose, glucose, sucrose, turanose and maltose can be purchased from the usual suppliers, as can melezitose, raffinose and isomaltose. Turanose can be obtained from Senn Chemicals of Dielsdorf, Switzerland, among others. See Reference (1) for retention times and separation of all honey sugars.

Pipette 25ml methanol into a 100 ml calibrated flask. Depending on the sugars to be analysed, dissolve the amounts detailed below in approximately 40ml water and transfer quantitatively to the flask and fill to the mark with water.

Fructose: 2.000 g

Glucose: 1.500 g

Sucrose: 0.250 g

Turanose: 0.150 g

Maltose: 0.150 g

Use a syringe and a pre-mounted membrane filter to transfer the solution to sample vials. The standard solutions are stable for 4 weeks in the refrigerator at 4^0 C and for six months at -18^0 C.

C.3 EQUIPMENT

Sample vials.

Ultrasonic bath.

Calibrated flasks, volume 100 ml. 25-ml-pipette.

Membrane filter for aqueous solutions, pore size 0.45 µm.

Filter holder for membrane filters with suitable syringe.

High Performance Liquid Chromatograph consisting of pump, sample applicator, temperature regulated RI-detector thermostated at 30^o C*, temperature regulated column oven at 30^0C, integrator.

Analytical stainless-steel column, e.g. 4.6 mm in diameter, 250 mm length, containing amine-modified silica gel with 5-7 µm particle size.

Before use, carry out a system suitability test to ensure all the sugars can be separated.

* Note: the chromatography can be carried out at room temperature without influence on the results of the sugars, determined by the present method. However under these conditions no separation of erlose and melezitose is possible (1).

C.4. PROCEDURE

Preparation of samples

If necessary, prepare the honey according to the section Sampling of INTRODUCTION AND GENERAL COMMENTS ON THE METHODS.

Preparation of the sample solution

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

High Performance Liquid Chromatography (HPLC)

If a column of the type described above is used, the following conditions have been found to give satisfactory separation.

Flow rate: 1.3 ml/min

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

Column and detector temperature: 30^0C

Sample volume: 10 µl

Note: If it is not possible to carry out the analysis at 300C and if the detector cannot be thermostated at 30^oC, carry out the analysis at ambient temperature. In this case it is not possible to separate melezitose and erlose.

Note: Identical volumes of sample and standard solution should be injected.

C.5 Calculation and Expression of Results

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.

The mass percentage of the sugars, W, to be determined of fructose, glucose, etc and maltose in g/100g is calculated according to the following formula (external standard procedure):

$$W = \frac{A1 \times V1 \times m1 \times 100}{A2 \times V2 \times mo}$$

Where

A1 - Peak areas or peak heights of the given sugar compound in the sample solution, expressed as units of area, length or integration.

A2 - Peak heights of the given sugar compound in the standard solution, expressed as units of area, length or integration.

V1 - Total volume of the sample solution in ml

V2 - Total volume of the standard solution in ml m1 = Mass amount of the sugar in grams in the total volume of the standard *mo* - sample weight in g the result is rounded to one decimal place.

C.6 PRECISION OF THE PROCEDURE

The parameters r and R were determined in a DIN ring trial (2).

Sample No.	Fructose g/100 g	r	R
1	3.21	0.8	1.6
2	42.4	0.9	2.3
3	37.9	1.0	1.6

NB r - repeatability and R - reproducibility

Sample No.	Glucose g/100 g	r	R
1	23.0	0.9	2.1
2	28.5	0.8	1.8
3	32.0	1.1	1.4

Sample No.	Sucrose g/100 g	r	R
1	-	-	-
2	-	-	-
3	2.8	0.4	0.9

Sample No.	Turanose g/100	r	R
1	2.1	0.4	0.8
2	1.7	0.3	0.5
3	1.3	0.3	0.8

Sample No.	Maltose g/100 g	r	R
1	4.8	0.5	2.5
2	2.0	0.6	1.3
3	2.3	0.5	0.7

The repeatability and reproducibility have been calculated from the results on three types of honey analyzed by all laboratories collaborating in the study.

Annex D
(Normative)
Determination of sucrose content by Feheling Solution

D.1 Principle of the method — Based on the Walker (1917) inversion method.

D.2 Reagents

D.2.1 Soxhlet's modification of Fehling's solution (C.1.2.1)

D.2.2 Standard invert sugar solution (C.1.2.2)

D.2.3 Hydrochloric acid (6.34 N aqueous)

D.2.4 Methylene blue solution 2 g/l (C.1.2.3)

D.2.5 Sodium hydroxide solution (5 N aqueous)

D.3 Preparation for analysis

The honey is prepared for sampling and subsequent analysis as in Clause C.1.3.

D.4 Procedure

D.4.1 Preparation of test sample

Prepare the honey sample as in C.1.4.1.1 a).

Dilute 10 mL of this solution to 250 mL with distilled water to give honey solution (for sucrose determination), or

Prepare the honey solution as indicated in C.1.4.1.2.

D.4.2 Hydrolysis of the test sample

Place 50 mL of the honey solution (D 4.1) in a 100-mL volumetric flask, together with 25-mL distilled water; heat the mixture to 65°C over a boiling water bath. The flask is then removed from the water bath and 10 mL of 6.34 N hydrochloric acid added. The solution is allowed to cool naturally for 15 min and then brought to 20 °C and neutralized with 5 N sodium hydroxide using litmus paper as indicator, cooled again, and the volume adjusted to 100 mL to give diluted honey solution.

D.4.3 Titration

Titration with the diluted honey solution (D 4.2) is carried out as in C.4.3 and C.4.4.

D 4.4 Calculation and expression of results

Calculate percent invert sugar (g invert sugar per 100 g honey) after inversion using the appropriate formula as for the determination of per cent invert sugar before inversion (C.4.5).

Sucrose content = (invert sugar content after inversion - (minus) invert sugar content before inversion) x 0.95.

The result is expressed as g apparent sucrose per 100 g honey.

Annex E
(Normative)
Determination of electrical conductivity

E.1 Apparatus

E.1.1 Conductivity meter, lower range 10^{-7}S

E.1.2 Conductivity cell, platinized double electrode (immersion electrode)

E.1.3 Thermometer with division to 0.1 ^{0}C

E.1.4 Water bath, thermostatically controlled at a temperature of 20 ^{0}C + 0.5 ^{0}C

E.1.5 Volumetric flask, 100ml and 1000ml

E.1.6 Beakers, tall form

E.2 Reagents

E.2.1 freshly distilled water

E.2.2 Potassium chloride solution, 0.1M

Dissolve 7.4557g of potassium chloride (KCl), dried at 130 ^{0}C, in freshly distilled water in a 1000ml flask and fill to volume with distilled water. Prepare fresh on the day of use.

E.3 Procedure

E.3.1 Determination of the cell constant

E.3.1.1 Transfer 40ml of the potassium chloride solution to a beaker. Connect the conductivity cell to the conductivity meter, rinse the cell thoroughly with the potassium chloride solution and immerse the cell in the solution, together with a thermometer. Read the electrical conductance of this solution in mS after the temperature has equilibrated to 20 ^{0}C.

N.B: Most conductivity meters are direct current. In order to avoid false results due to polarization effects, the measurement time should be as short as possible.

E.3.1.2 Calculate the cell constant K, using the following formula:

$$K = 11.691 \times 1/G$$

Where:

K = the cell constant in cm $^{-1}$

G = the electrical conductance in ms, measured with the conductivity cell.

11.691 = the sum of the conductivity of a value of the electrical conductivity of freshly distilled water in mS.cm^{-1} and the electrical conductivity of a 0.1M potassium chloride solution, at 20 ^{0}C.

E 3.1.3 Rinse the electrode thoroughly with distilled water after the determination of the cell constant.

E.3.2 Preparation of the sample solution

E.3.2.1 Dissolve an amount of honey, equivalent to 20.0g anhydrous honey, in distilled water. Transfer the solution quantitatively to a 100 ml volumetric flask and make up to volume with distilled water.

E.3.2.2 Pour 40 ml of the sample solution into a beaker and place the beaker in the thermo stated water bath at 20 ^{0}C. Rinse the conductivity cell thoroughly with the remaining part of the sample solution. Immerse the conductivity cell in the sample solution. Read the conductance in mS after temperature equilibrium has been reached.

N.B: If the determination is carried out at a different temperature, because of lack of thermo stated cell, then a correction factor can be used for calculation of the value at 20 ^{0}C.

- For temperature above 20 ^{0}C: subtract 3.2% of the value per ^{0}C
- For temperature below 20 ^{0}C: add 3.2% of the value per ^{0}C

E.3.2.3 Calculation and expression of results

– Calculate the electrical conductivity of the honey solution, using the following formula:

$S_H = K.G$

Where:

S_H - electrical conductivity of the honey solution in mS.cm^{-1};

K - cell constant in cm^{-1}, and

G - conductance in mS. Express the result to the nearest 0.01 mS cm^{-1}

Annex F
(Normative)
Determination of pH and of free acidity by titration to pH 8.31

1. PRINCIPLE

The sample is dissolved in water, the pH measured and the solution titrated with 0.1M sodium hydroxide solution to pH 8.30 (1 - 3).

2. REAGENTS

These must be of analytical quality.

Distilled, carbon dioxide - free water.

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

0.1M sodium hydroxide solution accurately standardized (e.g. Titrisol).

3. EQUIPMENT

pH meter, accurate to 0.01 units.

Magnetic stirrer.

Burette 10 ml, 25 ml or automatic titrator.

Beaker, 250 ml.

4. PROCEDURE

Calibration of the pH meter

The meter should be calibrated at pH 3.0, 7.0 and 9.0.

Sample preparation

Carry out according to the section sampling of introduction and general comments on the methods.

Determination

Ensure the sample is representative. 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. Titrate with 0.1M NaOH to pH 8.30 (a steady reading should be obtained within 120 sec of starting the titration; in other words, complete the titration within 2 minutes.). Record the reading to the nearest 0.2ml when using a 10ml burette and to 0.01ml if the automatic titrator has sufficient precision.

5. CALCULATION AND EXPRESSION OF RESULTS

pH - Report to two decimal places.

5.1 Free acidity, express as milliequivalents or millimoles acid/kg honey

= ml of 0.1M NaOH x 10. Express the result to one place of decimals.

5.2 Acidity (as gluconic acid), per cent by mass = $\frac{0.25 \times V}{M}$

Where

V is the number of ml of 0.1 M NaOH used in the neutralization of the test sample; and

M is the mass in g of the sample taken for the test.

6. PRECISION

The probability level is 95 %. This means that, on average, in carrying out 20 determinations, 1 outlier may be expected.

The precision of the method was determined in

a) Study in the United Kingdom (4)

Sample No	Acidity meq/Kg	r	R
1	7.0	2.7	8.5
2	6.5	2.9	6.2
3	13.5	2.0	7.1
4	13.5	2.6	7.1

The repeatability and reproducibility have been calculated from the results on four types of honey analyzed by all laboratories collaborating in the study.

b) Trial of the International Honey Commission

Sample No	pH	r	R
1	3.6	0.07	0.5
2	3.7	0.07	0.7
3	3.9	0.06	0.5
4	4.0	0.04	0.4
5	4.0	0.06	0.5
6	4.0	0.04	0.2
7	4.3	0.06	0.2

Sample No	Acidity meq/Kg	r	R
1	11.2	0.9	6.23
2	16.6	1.2	10.1
3	17.8	1.5	7.6
4	21.3	0.7	6.7
5	39.5	0.9	11.3
6	42.9	1.0	13.2
7	46.2	1.7	14.6

The repeatability and reproducibility have been calculated from the results on seven types of honey analysed by all laboratories collaborating in the study.

Annex G
(Normative)
Determination of diastase activity

G.1 Principal of the method

Based on the method of Schade et al. (1958) as modified by White et al. (1959) and Hardon (1961).

G.2 Reagents

G.2.1Iodine stock solution

Dissolve 20 g potassium iodide, analytical grade, in 30 ml – 40 ml water containing 22 g potassium iodine, analytical grade and dilute to1 litre with water.

G.2.2 Iodine Solution 0.000 7 N

Dissolve 20 g potassium iodide, analytical grade in 30 ml – 40-ml water in a 500 -volumetric flask. Add 5.0 ml iodine stock solution to make up to volume, make a fresh solution every two days.

G.2.3 Acetate buffer – pH 5.3 (1.59 m)

Dissolve 87 g sodium acetate crystals ($CH_3COCNa.3H_2O$) in 400-ml water, add about 10.5 ml glacial acetic acid in little water and make up 500 ml. Adjust the pH to 5.3 with sodium acetate or acetic acid as necessary, using a pH meter.

G.2.4 Sodium chloride solution 0.5 M

Dissolve 14.5 g sodium chloride, analytical grade, in boiled out distilled water and make up to 500 ml. The keeping time is limited by mould growth.

G.2.5 Starch solution

G.2.5.1 Use a starch with a blue value of 0.5 - 0.55 using a 1 cm cell, as determined by the method below (J2.5.3).

G.2.5.2 Preparation of Solution

Weigh out the amount of start which is equivalent to 2.0 g anhydrous starch. Mix with 90 ml of water in a 250 -ml conical flask. Bring rapidly to the boil, swirling the solution as much as possible heating over a thick wire gauze preferably with an asbestos centre. Boil gently for 3 min, cover and allow to cool spontaneously to room temperature.

Transfer to a 100 m l volumetric flask, place in a water bath at 40 °C to attain this temperature and make up to volume at 40 °C.

G.2.5.3 Method of determination of blue value of starch

The amount of starch equivalent to 1 g anhydrous starch is dissolved by the above method (J.2.5.2), cooled and 2.5 ml acetate buffer added before making up to 100 ml in a volumetric flask. To a 100-ml volumetric flask add 75-ml water, 1-ml N hydrochloric acid and 1.5 ml of 0.02 N iodine solutions.

Then add 0.5 ml of the starch solution and make up to volume with water, allow to stand for one hour in the dark and read in 1 cm using a spectrophotometer 660 nm against a blank cell containing all the ingredients except the starch solution. Reading on the absorbance scale = Blue Value

G.3 Apparatus

G.3.1Water bath at 40 ± 0.2 °C

G.3.2 Spectrophotometer to read 550 nm

G.4 Sampling

The honey sample is prepared as in Clause C.1.3 without any heating.

G.5 Procedure

G.5.1 Preparation of test samples

10.0 g honey is weighed into a 50-ml beaker and 5.0-ml acetate buffer solution added, together with 20 ml water to dissolve the sample. The sample is completely dissolved by stirring the cold solution. 3 ml sodium chloride solution is added to a 50-ml volumetric flask and the dissolved honey sample is transferred to this and the volume adjusted to 50 ml. This gives the test honey solution.

NOTE: It is essential that the honey should be buffered before coming into contact with sodium chloride.

G.5.2 Standardization of the starch solution

The starch solution is warmed to 40°C and 5 ml pipetted into 10 ml of water at 40°C and mixed well. 1 ml of this solution is pipetted into 10 ml 0.000 7 N iodine solution, diluted with 35 m l of water and mixed well. The colour is read at 660 n m against a water blank using a 1 c m cell. The absorbance should be 0.760 ± 0.020. If necessary, the volume of added water is adjusted to obtain the correct absorbance.

I.5.3 Absorbance determination

Pipette 10-ml honey solution into 50-ml graduated cylinder and place in 40°C ± 0.2°C water bath with a flask containing starch solution. After 15 minutes' pipette 5 m l starch solution, mix, and s tart stop- watch. At 5 min intervals remove 1-ml aliquots and add to 10.00 ml 0.000 7 N iodine solution. Mix and dilute to standard volume (J.5.2). Determine absorbance at 660 nm in spectrophotometer immediately using a 1 cm cell. Continue taking 1-ml aliquots at intervals until an absorbance of less than 0.235 is obtained.

G.6 Calculation and expression of results

The absorbance values obtained (J.5.3) are plotted against time (min) on a rectilinear paper. A straight line in drawn through at least three points on the graph to determine the time when the reaction mixture reaches an absorbance of 0. 235. Divide 300 by time in minutes to obtain the diastase number (DN). This number ex presses the diastase activity as ml of 1 % starch solution hydrolyzed by the enzyme quantity contained in 1 g of honey in 1 h at 40 °C. This diastase number corresponds with the Gothe-scale number. Diastase activity = DN = ml starch solution (1%)/g honey/h at 40 °C.

AnnexH
(Normative)
Determination of hydroxymethylfurfural

Part 1Determination of hydroxymethylfurfural by HPLC

1. PRINCIPLE

Hydroxymethylfurfural (HMF) is determined in a clear, filtered, aqueous honey solution using reverse phase HPLC equipped with UV detection. The signal is compared with those from standards of known concentration. This method is based on the work of Jeuring and Kuppers (1, 2).

2. REAGENTS

Mobile phase: water-methanol (90+10 by volume), both HPLC quality.

Standard solutions: 5-(hydroxymethyl-) furan-2-carbaldehyde (HMF), (e.g. Merck No. 820 678 or Fluka No. 55690), 1, 2, 5 and 10 mg /L aqueous solution. The solution should be prepared on the day of use.

Determination of standard HMF-content

The absorbance A of the prepared standard solution is determined using an UV spectrophotometer at 285 nm in 1 cm quartz cells with water in the blank cell. The concentration of the standard solutions can be calculated from the literature values for molar absorptivity, ε = 16830 or absorptivity, a cm 1 1% = 133.57 (3).

Concentration in mg/L = A/1*133.57*1,000

where A is the absorbance of the standard solution.

The calculated content must correspond to the specifications given by the supplier.

The standard has to be stored at 4 - 8 °C under nitrogen. It is extremely hygroscopic.

3. EQUIPMENT

Liquid chromatograph with UV detector and integrator.

Column: any column with C18-reversed phase material.

e.gHypersil ODS 5 µm, 125 x 4 mm or 250 x 4 mm.

Membrane filter, 0.45 µm (e.g. Dynagard).

4. PROCEDURE

Preparation of samples.

Carry out according to the section sampling of introduction and general comments on the methods.

Determination

Accurately weigh about 10 g of prepared honey sample into a 50 ml beaker. Dissolve the sample in approx. 25 ml of water and transfer quantitatively to a 50 ml volumetric flask. Dilute to 50 ml with water. Filter through a 0.45 µm membrane filter to provide a sample solution ready for chromatography. Conditions for chromatography flow rate1.0 ml/ minute quantity injected 20 µL of sample or standard solution. detection UV 285 nm; range: 0.2 AUFS.

5. CALCULATION AND EXPRESSION OF RESULTS

The HMF content of the sample is calculated by comparing the corresponding peak areas of the sample and those of the standard solutions, taking into account the dilution. There is a linear

relationship between the concentration and the area of the HMF peak. Results are expressed in mg/kg, to 1 decimal place.

6. PRECISION

The precision of the method was determined in a trial of the International Honey Commission. The repeatability and reproducibility have been calculated from the results on three types of honey analyzed by all laboratories collaborating in the study.

Sample No	HMF mg/kg	r	R
1	5.2	0.4	1.6
2	22.8	1.2	4.9
3	42.3	2.1	7.3

Part 2 Determination of hydroxymethylfurfural after White

1. PRINCIPLE

The determination of the 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 (1, 2).

2. REAGENTS

Carrez solution I: dissolve 15 g of potassium hexacyanoferrate(II), K4Fe(CN)6•3H2O in water and make up to 100 ml.

Carrez solution II: dilute 30 g of zinc acetate, Zn(CH3.COO)2.2H2O and make up to 100 ml.

Sodium bisulphite solution 0.20 g/100 g: dissolve 0.20 g of solid sodium hydrogen sulphite NaHSO3, (metabisulphite, Na2S2O5), in water and dilute to 100 ml. Prepare fresh daily.

3. EQUIPMENT

Spectrophotometer operating in a wavelength range including 284 and 336 nm. 1 cm quartz cells Vortex mixer.

Filter paper (general purpose).

4. PROCEDURE

Sample preparation.

carry out according to the section sampling of introduction and general comments on the methods.

Determination. Accurately weigh approximately 5g of honey into a 50 ml beaker. Dissolve the sample in approximately 25 ml of water and transfer quantitatively into a 50 ml volumetric flask. Add 0.5 ml of Carrez solution I and mix. Add 0.5 ml of Carrez solution II, mix and make up to the mark with water(a drop of ethanol may be added to suppress foam). Filter through paper; rejecting the first 10 ml of the filtrate. Pipette 5.0 ml in each of two 2 test tubes (18 x 150 mm). Add 5.0 ml of water to one of the test tubes and mix well (the sample solution). Add 5.0 ml of sodium bisulphite solution 0.2% to the second
test tube and mix well (the reference solution).

Dilution of sample and reference solutions is carried out as follows:

Additions to test-tube	Sample solution	Reference solution
Initial honey solution	5.0 ml	5.0 ml
Water	5.0 ml	0.2%
Sodium bisulphite solution	-	5.0 ml

Determine the absorbance of the sample solution against the reference solution at 284 and 336 nm in 10 mm quartz cells within one hour. If the absorbance at 284 nm exceeds a value of about 0.6, dilute the sample solution with water and the reference solution with sodium bisulphite solution to the same extent in order to obtain a sample absorbance low enough for accuracy. If dilution is necessary,

The Dilution ,D= Final volume of sample solution/ 10

5. CALCULATION AND EXPRESSION OF RESULTS

HMF in mg/kg = $(A_{284} - A_{336}) \times 149.7 \times 5 \times D/W$

Where: A_{284} = absorbance at 284 nm

A_{336} = absorbance at 336 nm

1497 = 126* 1000 * 1000/ 16830 *10* 5 = Constant

126 = molecular weight of HMF

16830 = molar absorptivity ε of HMF at λ= 284 nm

1000 = conversion g into mg

10 = conversion 5 into 50 ml

1000 = conversion g of honey into kg

5 = theoretical nominal sample weight

D = dilution factor, in case dilution is necessary

W = Weight in g of the honey sample.

Express results in mg/kg to 1 decimal place.

6. PRECISION

a)trials according to DIN (3)

Sample No.	HMF mg/Kg	r	R
1	15 – 21	1.5	4.5
2	48 – 64	1.7	5.2

The repeatability and reproducibility have been calculated from the results on two types of honey analysed by all laboratories collaborating in the study.

b)trial conducted by the International Honey Commission

Sample No.	HMF mg/Kg	r	R
1	3.8	0.9	2.3
2	22.3	1.2	3.9
3	42.1	2.2	4.4

NOTE

Some honeys, such as lime, may show strong absorbance at 284nm due to interfering substances. If these have an absorbance at 336nm different to that at 284nm, the result will be in error. This difficulty is normally overcome by using a double-beam spectrophotometer. In the absence of such an instrument, sample dilution may be tried. In this case the dilution factor must be taken into account when calculating the result. If dilution is too great to give adequate accuracy, an alternative method should be used.

Annex I
(Normative)
Determination of water insoluble solids content

1. PRINCIPLE

The insoluble matter is collected on a crucible of specified pore size and the dried residue is weighed after being washed free of soluble material.

2. EQUIPMENT

Analytical balance, to 0.1mg.

Sintered glass crucible,

Pore size 15 to 40 microns.

Drying oven at 135 ± 10 ^{0}C.

3. PROCEDURE

Accurately weigh approximately 20 grams of honey and dissolve in about 200 ml of water at about 80 ^{0}C. Mix well.

Dry a crucible in the oven and leave to obtain ambient temperature in a desiccator containing an efficient desiccant such as silica gel. Weigh the crucible.

Filter the sample solution through the crucible. Wash carefully and extensively with warm water until free from sugars. Check by adding to some filtrate in a test tube some 1% phloroglucinol in ethanol. Mix and run a few drops of concentrated sulphuric acid down the sides of the tube. Sugars produce a color at the interface.

Dry the crucible at 135 ^{0}C for an hour, cool in the desiccator and weigh. Return to the oven for 30 minute intervals until constant weight is obtained.

4. CALCULATION AND EXPRESSION OF RESULTS

$$\% \text{ Insoluble Matter in g/100 g} = m / m_1 \times 100$$

Where

m = mass of dried insoluble matter, and

m_1 = mass of honey taken

5. PRECISION

The precision of the method was determined in the UK collaborative study. The values are in g/100g

Mean, X	0.021	0.009	0.031	0.011
Repeatability (r)	0.016	0.016	0.023	0.010
Reproducibility (R)	0.021	0.016	0.023	0.026

Annex J
(Normative)
Fluorometric determination of proline in honey by high-performance liquidchromatography after precolumnderivatization with7-fluoro-4-nitrobenzo-2- oxa-1, 3-diazole (NBD-F)

1. Scope

This method is applicable for all honey types for the Determination of Proline.

2. Principle

Proline is extracted from honey by 0.1M borate buffer solution derivatized by NBD-F and determined by HPLC fluorescence detector.

3. Reagent

3.1 The L-proline(99%) standard for HPLC Calibration

3.2 7-fluoro-4-nitrobenzo-2- oxa-1,3-diazole (NBD-F) for derivatization

3.3 Sodium hydroxide 0.1M

3.4 Disodium ethylene diamine tetra acetic acid (EDTA

3.5 The 0.1 M borate buffer solution containing 0.001 M disodium ethylene diamine tetra acetic acid (EDTA)

3.6 HPLC grade tetrahydrofuran

3.7 Acetonitrile HPLC grade

3.8 Methanol HPLC grade

3.9 Water HPLC grade

4. Equipment

Homogenizer Vortex

4.1 Ultrasonic bath

4.2 Re-circulating water bath

4.3 HPLC system (Agilent 1200, USA) equipped with a G1321A fluorescence detector.

5. Preparation of standard solutions

Proline (100 mg) was put into 100 mL volumetric flask and dissolved in 30 mL 0.1 M borate buffer solution, and diluted with the same solvent to obtain stock solutions with a concentration of 1.0 mg/mL. The calibration curve was obtained by diluting the proline stock solutions with 0.1 M borate buffer solution to produce the following concentrations:100.0 µg/mL, 40.0 µg/mL, 10.0 µg/mL, 5.00 µg/mL, 2.50 µg/mL, 0.625 µg/mL and 0.15 µg/mL. Stock solutions were stored at 4°C and were not used after 3 months. Working solutions were freshly prepared before use.

6. Sample preparation

For proline extraction, honey samples (1.0 g) were dissolved in 20 mL borate buffer solution and treated with ultrasound for approximately 10 min. Sample solutions were transferred into a 50 mL brown volumetric flask and further diluted to 50 mL with borate buffer solution. The extraction solution was then filtered through a 0.2 µm nylon filter membrane.

7. Derivatization method

For derivatization for 200 µL of each calibration solution or extracted sample solution, 70 µL acetonitrile and 30 µL NBD-F solution (100 mM in acetonitrile, stored at -20°C, restored to room temperature before use) were consecutively added into a brown glass vial. The mixture was allowed to react for 12 min at 60^0C and then placed on ice, followed by termination of the derivatization

reaction by adjusting the medium to approximately pH 1.0 with 0.1 M ice-cold HCl (100 µL). An aliquot of 20 µL of the derivatized sample was injected into the HPLC instrument for analysis.

8. Instrumental condition

8.1 Column: Eclipse XDB C18 (150 mm×4.6 mm, 5µm) and C18 (250 mm×4.6 mm, 5µm)

8.2 Mobilphase: 0.1 M sodium acetate buffer solution (pH adjusted to 7.2 with acetic acid):methanol:tetrahydrofuran (900:95:5 respectively, v/v/v) as mobile phase A and methanol as mobile phase B

	A	**B**	**Maximum pressure**
0-0.5	100%	%	400
0.5-7	75%	25%	400
7-8	75%	25%	400
8-12	0%	100%	400
12-15	100%	0%	400

8.3 Flow rate 1mL/min

8.4 Column temperature 300C

8.5 Rune time 15min

8.6 Injection volume 20µL

8.7 Detector fluorescence excitation 470nm and emission 530nm

9. Quality control

The quality of proline analysis is controlled by using CRM and matrix spikeand the recovery shale be70-120%

10. Calculation

Proline in mg/kg honey $= \frac{(A_2 \times C_1 \times V)}{A_1 \times M}$

Where: A_1 Peak Area of proline standard

A_2 Peak Area of Honey sample

V Dilution volume

M Mass of sample taken

Annex K
(Normative)
Sampling of extracted honey

K.1 General Requirements

In drawing, preparing, storing and handling samples, the following precautions and directions shall be observed.

K.1.1 Samples shall be taken in a protected place not exposed to damp air, dust or soot.

K.1.2 The sampling instrument shall be clean and dry when used.

K.1.3 Precautions shall be taken to protect the samples, the material being sampled, the sampling instrument and the containers for samples from adventitious contamination.

K.1.4 The samples shall be placed in clean and dry glass containers. The sample containers shall be of such a size that they are almost completely filled by the sample.

K.1.5 Each container shall be sealed air-tight after filling and marked with full details of sampling, code number and other important particulars of the consignment.

K.1.6 Samples shall be stored in such a manner that the temperature of the material does not vary unduly from the normal temperature.

K.2 Scale of sampling

K.2.1 Lot

All the containers in a single consignment belonging to the same grade of material shall constitute a lot. If the consignment is declared to consist of different grades of material, the containers belonging to the same grade shall be grouped together and the groups of containers of the same grade in a consignment shall constitute separate lots.

K.2.1.1 Samples shall be tested from each lot for ascertaining its conformity to the requirements of this specification.

K.2.2 The number of containers to be selected from each lot shall depend on the size of the lot and shall be done in accordance with Table 1.

Table 1 Number of containers to selected for sampling

Lot size (N)	Number of containers to be selected (n) for size of the container	
	500 g and above	Below 500 g
Up to 25	3	6
26 to 150	4	6
151 to 500	5	9
501 and above	7	12

K.2.3 The containers shall be chosen at random from the lot and for this purpose a random number table as agreed to between the purchaser the supplier shall be used. If such a table is not available, the following procedure shall be adopted:

Starting from any container in the lot, count them as 1, 2, 3..., up to *r* in a systematic manner, where *r* is equal to the integral part of *N*/*n*, *N* being the total number of containers in the lot, *n* the number of containers to be chosen (see Table 2). Every r^{th} container thus counted shall be separated until the requisite number of containers is obtained from the lot to give samples for test.

K.3 Test samples and referee samples

The samples shall be drawn and prepared according to M.3.1 and M.3.2, when the containers are selected according to col.2 Table 2. Clause M.3.3 shall be followed when the containers are selected according to col. 3 to Table 2.

K.3.1 Preparation of individual samples

Draw with suitable sampling instrument equal quantities of the material from different parts (top, middle, bottom, etc) of the container till about 300 g of the material is drawn; divide it into three equal parts. Each part so obtained shall constitute an individual sample representing the container and shall be transferred immediately to thoroughly cleaned, dry containers, sealed air-tight, and marked with particulars given under M.1.5. Three individual samples so obtained from each container shall be made into sets in such a way that each set has a sample representing each selected container. One of these shall be marked for the purchaser, another for the vendor and the third for the referee.

K.3.2 Preparation of composite sample

From the material from each of the selected container, remaining after the individual sample has been taken, approximately equal quantities of material shall be taken and mixed together so as to form a composite sample weighing about 150 g. This composite sample shall be divided into three equal parts and transferred to clean and dry containers, sealed air-tight and labelled with particulars as given in M.1.5. One of these composite samples shall be for the purchaser, another for the vendor and the third for the referee.

K.3.3 When honey is in containers of size less than 5 00 g, the number of containers shall be selected according to col.3 of Table 2. The selected containers shall be divided at random into three equal sets. The containers belonging to each set shall be opened and approximately equal quantity of material shall be taken and mixed together to form a composite sample of 50 g. The honey left in each container after the preparation of composite s ample shall be sealed air tight with all the particulars as given in M.1.5. The individual samples also shall be packed in air-tight containers with all the particulars as given in M.1.5. The three sets of the individual sample with their corresponding composite sample shall be marked in such a way that one set is for the purchaser, another for the vendor and the third for the referee.

K.3.4 Referee sample

Referee sample shall consist of a set of individual samples (M.3.1 and M.3.3) and a composite sample (M.3.2 and M.3.3) marked for this purpose and shall bear the seals of the purchaser and the vendor. These shall be kept at a place as agreed to between the two.

K.4 Number of tests

K.4.1 Tests for the moisture, ash, total reducing sugars and Fiehe's test shall be conducted on each of the samples constituting a set of individual samples.

K.4.2 Test for specific gravity, sucrose percent, fructose-glucose ratio and acidity s hall be conducted on the composite sample.

K.4.2.1 If Fiehe's test is positive, determination of hydroxyl methyl furfural (HMF) content shall be carried out on the individual sample.

K.5 Criteria for conformity

K.5.1 A lot shall be declared to have satisfied the requirements of the specification when M.5.1.1and M.5.1.4 are satisfied.

K.5.1.1 Each individual sample shall satisfy the requirements given in M .1.1, 1.2 and 1.3.

K.5.1.2 The test results on the composite sample for characteristics mentioned in M.4.2 and M.4.2.1shall

satisfy the corresponding requirements as given in Table 2.

K.5.1.3 The test result on the individual samples for Fiehe's test shall be negative. The HMF content determination, carried out on t hose individual samples in which Fiehe's test is positive, shall satisfy the corresponding requirements as given in Table 2.

N.5.1.4 The test results for moisture, ash and total reducing sugars shall be recorded as shown in Table 2. The mean and range for the test results of the individual sample shall be calculated as follows:

$$\text{Mean (X)} = \frac{Sum of test results}{Number of test results}$$

Range (R) = the difference between the maximum and minimum values of the test results

The mean and range shall be recorded as shown in col. 3 and 4 of Table 2 respectively. The appropriate expression as shown in col. 6 of Table 2 shall be calculated. If the value of these expressions satisfies the relevant conditions given in col. 6 of Table 2, the lot shall be declared to have satisfied the requirements of moisture, ash and total reducing sugars.

Table 2 Criteria for conformity of result

Characteristic	**Test results 1, 2, ...,**	**Average**	**Range**	**Criteria for conformity**
Moisture	—	X1	R1	X1 + 0.6R1 ≤ the value of that grade as specified in Table 1
Ash	—	X2	R2	X2 + 0.6R2 ≤ 0.5
Total reducing sugars	—	X3	R3	X3 - 0.6R3 ≥ the value of that grade as specified in Table 1

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INSTITUTE OF ETHIOPIAN STANDARDS

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For More Information?

Contact us at the following address.

The Head Office of IES is at Addis Ababa.

☎***011-6460685, 011-6460565***
011-6460880
✉***2310AddisAbaba, Ethiopia***
E-mail:info@ethiostandards.org
Website:www.ethiostandards.org

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