MANUAL OF METHODS OF

ANALYSIS OF FOODS

HONEY& OTHER BEE HIVE

PRODUCTS

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Note: The test methods given in the manual are standardized / validated and were taken from national or international methods or recognized specifications, however it would be the responsibility of the respective testing laboratory to verify the performance of these methods onsite and ensure that it gives proper results before putting these methods in to use.

A. Honey

FOOD SAFETY AND STANDARDS AUTHORITY OF INDIA Inspiring Trust, Assuring Safe & Nutritious Food Ministry of Health and Family Welfare, Government of India	**Determination of Specific gravity**		
Method No.	FSSAI 04B.001:2023	**Revision No. & Date**	0.0
Scope	All types of Honey including Carvia Callosa and Honey dew		
Caution	1. Honey sample must be kept at moisture free place in air tight jar. 2. Mix the sample thoroughly before taking test portion for analysis.		
Principle	Specific gravity is the ratio of the density of a substance to that of a standard substance**.** Specific gravity of honey calculated by the ratio of weight of a given volume of the honey at 27±1°C to the weight of an equal volume of water at 27±1°C with the help of Specific gravity bottle.		
Apparatus/Instruments	1. Specific gravity bottle. 2. Thermostatically controlled water bath-maintained at 27±1°C. 3. Weighing balance. 4. Sieve (No. 40)		
Materials and Reagents	NA		
Preparation of Reagents	NA		
Sample Preparation	A) **Liquid or Strained honey**: If honey is free from granulation, mix thoroughly by stirring or shaking before weighing portion. If granulated, place closed sample container in water bath without submerging and heat at sample at 60°C for 30 min until liquefied. Occasional shaking is essential. cool the honey sample rapidly as soon it liquefies and mix thoroughly before taking test portion for determination. **Do not heat honey sample intended for diastase determination**. If foreign matter such as wax, sticks, bees, particles of comb etc. is present, heat honey to 40°C and filter through cheesecloth in hot water funnel before weighing, test portions for analysis. B) **Comb Honey**: Cut across top of comb, if sealed and separate completely from comb by straining through No. 40 sieve. When portions of comb or wax pass through sieve, heat product as in A and filter through cheese cloth. If honey is granulated in comb, heat sample until wax is liquefied and after this stir, cool, and remove wax.		
Method of analysis	1. Clean and thoroughly dry the specific gravity bottle and weigh. 2. Fill it up to the mark with freshly boiled and cooled distilled water which has been maintained at 27 ± 1°C and weigh. 3. Remove the water, dry bottle again and fill it with the honey sample maintained at the same temperature. 4. Weigh the bottle again.		
Calculation with units of expression	Specific gravity at 27 °C = $\frac{C - A}{B - A}$ Where C = mass, in g, of the specific gravity bottle with the honey sample;		

	A = mass, in g, of the empty specific gravity bottle; and B = mass, in g, of the specific gravity bottle with water
Inference (Qualitative Analysis)	NA
Reference	IS 4941:1994 AOAC (920.180) 21st edition-2019
Approved by	Scientific Panel on Methods of Sampling and Analysis

FOOD SAFETY AND STANDARDS AUTHORITY OF INDIA Inspiring Trust, Assuring Safe & Nutritious Food Ministry of Health and Family Welfare, Government of India	**Determination of Moisture (Vacuum Oven Drying Method)**		
Method No.	FSSAI 04B.002:2023	**Revision No. & Date**	0.0
Scope	All types of Honey including Carvia Callosa and Honey dew.		
Caution	1. Honey sample must be kept at moisture free place in air tight jar. 2. Mix the sample thoroughly before taking test portion for analysis.		
Principle	Honey sample is heated in a vacuum oven under controlled conditions of pressure and temperature to remove moisture by passing dry air. Sample is weighed before and after drying to estimate moisture.		
Apparatus/Instruments	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. 2. Sand- Passing through 500-microns IS Sieve but retained on 180-micron IS Sieve. It shall be prepared by digestion with concentrated hydrochloric acid, followed by thorough washing with water till free form chlorides. It shall be dried and ignited to dull red heat. 3. Vaccum Oven		
Materials and Reagents	NA		
Preparation of Reagents	NA		
Sample Preparation	A) **Liquid or Strained honey**: If honey is free from granulation, mix thoroughly by stirring or shaking before weighing portion. If granulated, place closed sample container in water bath without submerging and heat at sample at 60°C for 30 min until liquefied. Occasional shaking is essential. cool the honey sample rapidly as soon it liquefies and mix thoroughly before taking test portion for determination. **Do not heat honey sample intended for diastase determination**. If foreign matter such as wax, sticks, bees, particles of comb etc. is present, heat honey to 40°C and filter through cheesecloth in hot water funnel before weighing, test portions for analysis. B) **Comb Honey**: Cut across top of comb, if sealed and separate completely from comb by straining through No. 40 sieve. When portions of comb or wax pass through sieve, heat product as in A and filter through cheese cloth. If honey is granulated in comb, heat sample until wax is liquefied and after this stir, cool, and remove wax.		
Method of analysis	1. Heat the dish containing 20 g of the prepared sand and a stirring rod in the oven for one hour. 2. Allow to cool in a desiccator for 30-40 mins. 3. Weigh accurately 2 g of the material into the tared dish. 4. Add 5 mL of distilled water in 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. 5. Place the dish on boiling water-bath for 30 mins. 6. Wipe the bottom of the dish and transfer it with the glass rod, to the vaccum oven maintained at a temperature between 60 °C and 70 °C and at a pressure not more than 50 nm of mercury.		

	7. After 2 h, remove the dish and transfer to a desiccator, allow it to cool and then weigh. 8. Replace the dish in the oven for a further period of one hour, remove and transfer to desiccator, cool and weigh again. Repeat the process of heating, cooling and weighing after every hour till consecutive weighing do not differ by more than 0.5 mg.
Calculation with units of expression	Moisture, % by mass = $\frac{100\ (M_1 - M_2)}{M_1 - M}$ Where M_1 = mass, in g, of the contents of the dish before drying M_2 = mass, in g, of the contents of the dish after drying M = mass, in g, of the empty dish with the sand and the glass rod.
Inference (Qualitative Analysis)	NA
Reference	IS 4941:1994 AOAC (920.180) 21st edition-2019
Approved by	Scientific Panel on Methods of Sampling and Analysis

FOOD SAFETY AND STANDARDS AUTHORITY OF INDIA Inspiring Trust, Assuring Safe & Nutritious Food Ministry of Health and Family Welfare, Government of India	**Determination of Moisture (By Refractometer)**		
Method No.	FSSAI 04B.003:2023	**Revision No. & Date**	0.0
Scope	All types of Honey including Carvia Callosa and Honey dew.		
Caution	1. Honey sample must be kept at moisture free place in air tight jar. 2. Mix the sample thoroughly before taking test portion for analysis. 3. Ensure that the prism of the refractometer is clean and dry .		
Principle	The method is based on the principle that refractive index increases with solids content. The moisture content value is determined from the refractive index of the honey by reference to a standard table.		
Apparatus/Instruments	Refractometer		
Materials and Reagents	NA		
Preparation of Reagents	NA		
Sample Preparation	A) **Liquid or Strained honey**: If honey is free from granulation, mix thoroughly by stirring or shaking before weighing portion. If granulated, place closed sample container in water bath without submerging and heat at sample at 60°C for 30 min until liquefied. Occasional shaking is essential. cool the honey sample rapidly as soon it liquefies and mix thoroughly before taking test portion for determination. **Do not heat honey sample intended for diastase determination**. If foreign matter such as wax, sticks, bees, particles of comb etc. is present, heat honey to 40°C and filter through cheesecloth in hot water funnel before weighing, test portions for analysis. B) **Comb Honey**: Cut across top of comb, if sealed and separate completely from comb by straining through No. 40 sieve. When portions of comb or wax pass through sieve, heat product as in A and filter through cheese cloth. If honey is granulated in comb, heat sample until wax is liquefied and after this stir, cool, and remove wax.		
Method of analysis	1. Clean and dry the refractometer before use. 2. Determine the refractometer reading of honey at 20 °C and calculate the percentage of moisture from the values given in Table 1. 3. If the determination is made at a temperature other than 20^0C, correct the reading according to the Note in Table 1.		
Calculation with units of expression	Table 1. Relationship Between Refractive Index and Moisture Content of Honey		

Refractive Index @20 ° C	Moister	Refractive Index @20 ° C	Moister
1.504 4	13.0	1.488 5	19.2
1.503 8	13.2	1.488 0	19.4
1.503 3	13.4	1.487 5	19.6
1.502 8	13.6	1.487 0	19.8
1.502 3	13.8	1.486 5	20.0
1.501 8	14.0	1.486 0	20.2

		1.501 2 14.2 1.500 7 14.4 1.500 2 14.6 1.499 7 14.8 1.499 2 15.0 1.498 7 15.2 1.498 2 15.4 1.497 6 15.6 1.497 1 15.8 1.496 6 16.0 1.496 1 16.2 1.495 6 16.4 1.495 1 16.6 1.494 6 16.8 1.494 0 17.0 1.493 5 17.2 1.493 0 17.4 1.492 5 17.6 1.492 0 17.8 1.491 5 18.0 1.491 0 18.2 1.490 5 18.4 1.490 0 18.6 1.489 5 18.8 1.489 0 19.0	1.485 5 20.4 1.485 0 20.6 1.484 5 20.8 1.484 0 21.0 1.483 5 21.2 1.483 0 21.4 1.482 5 21.6 1.482 0 21.8 1.4815 22.0 1.4810 22.2 1.480 5 22.4 1.480 0 22.6 1.479 5 22.8 1.479 0 23.0 1.478 5 23.2 1.478 0 23.4 1.477 5 23.6 1.477 0 23.8 1.476 5 24.0 1.476 0 24.2 1.475 5 24.4 1.475 0 24.6 1.474 5 24.8 1.474 0 25.0
		NOTE - Temperature correction for refractive index = 0.000 23 per ^{0}C. If the reading is made at a temperature above 20°C, add the correction; if made below, subtract the correction	
Inference (Qualitative Analysis)	NA		
Reference	IS 4941:1994 AOAC (920.180)21st edition-2019		
Approved by	Scientific Panel on Methods of Sampling and Analysis		

FOOD SAFETY AND STANDARDS AUTHORITY OF INDIA Inspiring Trust, Assuring Safe & Nutritious Food Ministry of Health and Family Welfare, Government of India	**Determination of Total Reducing Sugars, Sucrose And Fructose-Glucose Ratio (Titrimetric Method)**		
Method No.	FSSAI 04B.004:2023	**Revision No. & Date**	0.0
Scope	All types of Honey including Carvia Callosa and Honey dew		
Caution	1. Honey sample must be kept at moisture free place in air tight jar. 2. Mix the sample thoroughly before taking test portion for analysis. 3. Always wear gloves and mask while doing sample analysis.		
Principle	This method is the modification of the Lane and Eynon procedure, involving the reduction of Soxhlet's modification of Fehling's solution by titration at boiling point against a solution of reducing sugar in honey by using methylene blue as internal indicator. The difference in the concentrations of invert sugar multiplied by 0.95 to give the apparent sucrose content.		
Apparatus/Instruments	1. Weighing balance 2. Volumetric Flask-250 mL 3. Volumetric Flask-1000 mL 4. Burrete-50 mL		
Materials and Reagents	1. Copper Sulphate Solution (Solution A) 2. Potassium Sodium Tartrate (Rochelle Salt) (Solution B) 3. Hydrochloric Acid (12 N) 4. Standard Invert Sugar Solution 5. Methylene Blue Indicator		
Preparation of Reagents	1. **Soxhlet Modification of Fehling's Solution**- Prepare by mixing equal volumes of Solution A and solution B immediately before using. 2. **Copper Sulphate Solution (Solution A)** - Dissolve 34.639 g copper sulphate crystals ($CuSO_4.5H_2O$) in 500 mL distilled water, and filter through glass wool or filter paper. 3. **Standardization of Copper Sulphate Solution**- Using separate pipette, pipette out accurately 5 mL of Solution A and 5 mL of Solution B into a conical flask of 250 mL capacity. Heat this mixture to boiling on an asbestos gauze and add standard invert sugar solution from a burette, about one millilitre less than the expected volume which will reduce the Fehling's solution completely (about 48 mL). Add one millilitre of methylene blue indicator while keeping the solution boiling. Complete the titration within three minutes, the end point being indicated by change of color from blue to red. From the volume of invert sugar solution used, calculate the strength of the copper sulphate solution by multiplying the titrate value by 0.001 (mg/ml of the standard invert sugar solution). This would give the quantity of invert sugar required to reduce the copper in 5 ml of copper sulphate solution. 4. **Potassium Sodium Tartrate (Rochelle Salt) Solution (Solution B)**- Dissolve 173 g of potassium sodium tartrate and 50 g of sodium hydroxide in water and makeup volume to 500 mL. Let the solution stand for a day and filter. **5. Hydrochloric Acid- Sp gr 1.18 at 20 °C (approximately 12 N)** 6. **Standard Invert Sugar Solution**- Weigh accurately 0.95 g sucrose and dissolve it in 500 mL of water. Add 2 mL of concentrated hydrochloric acid, boil gently for 30 mins and keep aside for 24 h. then neutralize with sodium carbonate and make the final volume to 1000 mL. 50 mL of this solution contains 0.05 g invert sugar. 7. **Methylene Blue indicator**- 0.2 percent in water.		

	Reagents for Fructose-Glucose Ratio 1. Iodine Solution- 0.05 N 2. Sodium Hydroxide Solution- 0.1 N 3. Sulphuric acid- concentrated 4. Standard Sodium Thiosulphate Solution- 0.05 N.
Sample Preparation	A) **Liquid or Strained honey**: If honey is free from granulation, mix thoroughly by stirring or shaking before weighing portion. If granulated, place closed sample container in water bath without submerging and heat at sample at 60°C for 30 min until liquefied. Occasional shaking is essential. cool the honey sample rapidly as soon it liquefies and mix thoroughly before taking test portion for determination. **Do not heat honey sample intended for diastase determination**. If foreign matter such as wax, sticks, bees, particles of comb etc. is present, heat honey to 40°C and filter through cheesecloth in hot water funnel before weighing, test portions for analysis. B) **Comb Honey**: Cut across top of comb, if sealed and separate completely from comb by straining through No. 40 sieve. When portions of comb or wax pass through sieve, heat product as in A and filter through cheese cloth. If honey is granulated in comb, heat sample until wax is liquefied and after this stir, cool, and remove wax.
Method of analysis	**Procedure for Total Reducing Sugar** 1. Place one gram (W) of the prepared sample of honey into 250-mL volumetric flask and dilute with 150 mL of water. 2. Mix thoroughly the contents of the flask and make the volume to 250 mL with water. 3. Using separate pipettes, take accurately 5 mL each of solution A and solution B, in a porcelain dish or in conical flask. 4. Add 12 mL of honey solution from burette and heat to boiling over asbestos gauze. 5. Add one millilitre of methylene blue indicator and while keeping the solution boiling complete the titration, within three minutes. 6. The end point being indicated by change of color from blue to red. 7. Note the volume (H) in mL of honey solution required for the titration. **Procedure for Sucrose** 1. To 100 mL of the stock honey solution add one millilitre 1.0 millilitre of concentrated hydrochloric acid and heat the solution to near boiling. 2. Keep aside overnight. Neutralize this inverted honey solution with sodium carbonate and determine the total reducing sugars as described. **Procedure for Fructose-Glucose Ratio** 1. Pipette 50 mL of honey solution in a 250 mL stopped flask. 2. Add 40 mL of iodine solution and 25 mL of sodium hydroxide solution. 3. Acidify with 5 mL of sulphuric acid and titrate quickly the excess of iodine against standard sodium thiosulphate solution. 4. Conduct a blank using 50 mL of water instead of honey solution.
Calculation with units of expression	Total reducing sugar, percent by mass = $\frac{250 \times 100 \times S}{H \times M}$ Where S = strength of copper sulphate solution; H = volume, in ml, of honey solution required for titration; and M = mass, in g, of honey

	Calculation for Sucrose Sucrose, percent by mass = [(reducing sugars after inversion, percent by mass) – (reducing sugars before inversion, percent by mass)] x 0.95 **Calculation for Fructose-Glucose Ratio** $\text{Approximate glucose, percent by mass (w)} = \frac{(B - S) \times 0.004502 \times 100}{a}$ where B = volume of sodium thiosulphate solution required for the blank, S = volume of sodium thiosulphate solution required for the sample, and a = mass of honey taken for test. $\text{Approximate fructose, percent by mass (x)} = \frac{\text{Approximate total reducing Sugar, percent - w}}{0.925}$ $\text{True glucose, percent by mass (y)} = w - 0.012 x$ $\text{True fructose, percent by mass (z)} = \frac{\text{Approximate reducing sugars, percent - y}}{0.925}$ True reducing sugars, percent by mass = y + z $\text{Fructose-glucose ratio} = \frac{\text{True fructose, percent by mass (z)}}{\text{True glucose, percent by mass (y)}}$
Inference (Qualitative Analysis)	NA
Reference	IS 4941:1994 AOAC (920.180)21st edition-2019, IHC(2009)
Approved by	Scientific Panel on Methods of Sampling and Analysis

FOOD SAFETY AND STANDARDS AUTHORITY OF INDIA Inspiring Trust, Assuring Safe & Nutritious Food Ministry of Health and Family Welfare, Government of India	**Determination of Sucrose and F/G Ratio (HPLC Method)**		
Method No.	FSSAI 04B.005:2023	**Revision No. & Date**	0.0
Scope	All types of Honey including Carvia Callosa and Honey dew		
Caution	1. Honey sample must be kept at moisture free place in air tight jar. 2. Mix the sample thoroughly before taking test portion for analysis. 3. Always wear gloves and mask while doing sample analysis.		
Principle	Weighed sample dissolved in water and diluted with Acetonitrile, injected on HPLC-RID for the separation and quantification of sugars.		
Apparatus/Instruments	1. Liquid chromatography- equipped with Refractive Index Detector (RID) 2. Column- 300 x 4 (id) mm µ-Bondapak or Carbohydrate or equivalent 3. Syringe filters: 0.45 µm filters stable in organic solvents		
Materials and Reagents	1. Acetonitrile 2. Ultra pure water		
Preparation of Reagents	1. Mobile phase- LC grade acetonitrile diluted with ultra pure water (83 + 17): Degas mobile phase daily by magnetic stirring 15 min under vaccum. 2. Sugar standard solutions- Weigh 3.804 g fructose, 3.10 g glucose, and 0.602 g sucrose standards in 100 mL volumetric flask and dissolve in 50 mL water and make up the volume with Acetonitrile.		
Sample Preparation	A) **Liquid or Strained honey**: If honey is free from granulation, mix thoroughly by stirring or shaking before weighing portion. If granulated, place closed sample container in water bath without submerging and heat at sample at 60°C for 30 min until liquefied. Occasional shaking is essential. cool the honey sample rapidly as soon it liquefies and mix thoroughly before taking test portion for determination. **Do not heat honey sample intended for diastase determination**. If foreign matter such as wax, sticks, bees, particles of comb etc. is present, heat honey to 40°C and filter through cheesecloth in hot water funnel before weighing, test portions for analysis. B) **Comb Honey**: Cut across top of comb, if sealed and separate completely from comb by straining through No. 40 sieve. When portions of comb or wax pass through sieve, heat product as in A and filter through cheese cloth. If honey is granulated in comb, heat sample until wax is liquefied and after this stir, cool, and remove wax.		
Method of analysis	1. Weigh 5.0 g test portion in small beaker and transfer to 50 mL volumetric flask with 25 mL water. 2. Immediately dilute to volume (to make final volume 50mL) with Acetonitrile. 3. Filter through 0.45 µm filter. 4. Inject 10 µl standard solutions into instrument and establish retention times, measure peak heights, and check reproducibility. Repeat same for test solution. 5. Run Time: 20 min 6. Flow rate: 1.0 ml/min (3.45 Mpa; ca 500 psi); 7. Column temperature: ambient (ca 23 °C)		
Calculation with units of expression	Calculate glucose, fructose, and sucrose from integrator values or from peak heights as follows: Weight percent sugar = 100 x (PH/PH') x (V/V') x (W'/W)		

	Where PH and PH' = peak heights (or integrator values) of test solution and standard, respectively; V and V' = ml test and standard (50 and 100) solutions, respectively; and W and W' = g test portion (5.000) and standard, respectively.
Inference (Qualitative Analysis)	NA
Reference	AOAC 977.20
Approved by	Scientific Panel on Methods of Sampling and Analysis

	Determination of Total Ash		
Method No.	FSSAI 04B.006:2023	**Revision No. & Date**	0.0
Scope	All types of Honey including Carvia Callosa and Honey dew		
Caution	1. Honey sample must be kept at moisture free place in air tight jar. 2. Mix the sample thoroughly before taking test portion for analysis. 3. Always wear gloves and mask while doing sample analysis.		
Principle	The honey is ashed at a temperature 600°C ± 20 and the residue weighed.		
Apparatus/Instruments	1. Muffle -Furnace 2. Silica Crucible		
Materials and Reagents	NA		
Preparation of Reagents	NA		
Sample Preparation	A) **Liquid or Strained honey**: If honey is free from granulation, mix thoroughly by stirring or shaking before weighing portion. If granulated, place closed sample container in water bath without submerging and heat at sample at 60°C for 30 min until liquefied. Occasional shaking is essential. cool the honey sample rapidly as soon it liquefies and mix thoroughly before taking test portion for determination. **Do not heat honey sample intended for diastase determination**. If foreign matter such as wax, sticks, bees, particles of comb etc. is present, heat honey to 40°C and filter through cheesecloth in hot water funnel before weighing, test portions for analysis. B) **Comb Honey**: Cut across top of comb, if sealed and separate completely from comb by straining through No. 40 sieve. When portions of comb or wax pass through sieve, heat product as in A and filter through cheese cloth. If honey is granulated in comb, heat sample until wax is liquefied and after this stir, cool, and remove wax.		
Method of analysis	1. Weigh accurately 5 g to 10 g of the honey sample in a silica or platinum dish, 2. Add a few drops of pure olive oil to prevent spattering, heat carefully over a low flame until swelling ceases. 3. Ignite in a muffle furnace at 600 ± 20 °C till white ash is obtained. 4. Cool the dish in a desiccator and weigh. 5. Incinerate to constant weight.		
Calculation with units of expression	Ash, percent by mass = $\frac{100\ (M2 - M)}{M1 - M}$ Where M2 = mass, in g, of the dish with the ash; M = mass, in g, of the empty dish; and M1 = mass, in g, of the dish with the material taken for the test.		
Inference (Qualitative Analysis)	NA		

Reference	IS 4941:1994 AOAC (920.180)21st edition-2019
Approved by	Scientific Panel on Methods of Sampling and Analysis

	Determination of Acidity as Formic acid		
Method No.	FSSAI 04B.007:2023	**Revision No. & Date**	0.0
Scope	All types of Honey including Carvia Callosa and Honey dew		
Caution	1. Honey sample must be kept at moisture free place in air tight jar. 2. Mix the sample thoroughly before taking test portion for analysis. 3. Always wear gloves and mask while doing sample analysis.		
Principle	The acidity is obtained by adding an excess of sodium hydroxide to the honey solution and developed pink color of Phenolphthalein indicator observed as end point.		
Apparatus/Instruments	1. Burette 2. Conical Flask-50 mL		
Materials and Reagents	1) Standard Sodium Hydroxide Solution- 0.05 N. 2) Phenolphthalein Indicator Solution		
Preparation of Reagents	1) Standard Sodium Hydroxide Solution- 0.05 N. 2) Phenolphthalein Indicator Solution- Dissolve 0.5 g of Phenolphthalein in 100 mL of 50 percent ethyl alcohol (v/v)		
Sample Preparation	A) **Liquid or Strained honey**: If honey is free from granulation, mix thoroughly by stirring or shaking before weighing portion. If granulated, place closed sample container in water bath without submerging and heat at sample at 60°C for 30 min until liquefied. Occasional shaking is essential. cool the honey sample rapidly as soon it liquefies and mix thoroughly before taking test portion for determination. **Do not heat honey sample intended for diastase determination**. If foreign matter such as wax, sticks, bees, particles of comb etc. is present, heat honey to 40°C and filter through cheesecloth in hot water funnel before weighing, test portions for analysis. B) **Comb Honey**: Cut across top of comb, if sealed and separate completely from comb by straining through No. 40 sieve. When portions of comb or wax pass through sieve, heat product as in A and filter through cheese cloth. If honey is granulated in comb, heat sample until wax is liquefied and after this stir, cool, and remove wax.		
Method of analysis	1) Take 10 g of the sample in a suitable titration flask and dissolve it in 75 mL of carbon dioxide-free water. 2) Mix thoroughly. 3) Titrate against standard sodium hydroxide solution using 4 to 6 drops of carefully neutralized phenolphthalein solution (pink color of indicator should persist for at least 10 seconds). 4) Determine blank on water with indicator and correct the volume of standard sodium hydroxide solution used.		
Calculation with units of expression	Acidity (as Formic acid), percent by mass = $\frac{0.23 \times V}{M}$		

	Where V = corrected volume of 0.05 N sodium hydroxide solution required for titration; and M = mass, in g, of the sample taken for the test.
Inference (Qualitative Analysis)	NA
Reference	IS 4941:1994 AOAC (920.180) 21st edition-2019
Approved by	Scientific Panel on Methods of Sampling and Analysis

FOOD SAFETY AND STANDARDS AUTHORITY OF INDIA Inspiring Trust, Assuring Safe & Nutritious Food Ministry of Health and Family Welfare, Government of India	**Determination of Free Acidity**		
Method No.	FSSAI 04B.008:2023	**Revision No. & Date**	0.0
Scope	All types of Honey including Carvia Callosa and Honey dew		
Caution	1. Honey sample must be kept at moisture free place in air tight jar. 2. Mix the sample thoroughly before taking test portion for analysis. 3. Always wear gloves and mask while doing sample analysis.		
Principle	The free acidity is the acidity titratable with sodium hydroxide up to the equivalence point.		
Apparatus/Instruments	1. Burette		
Materials and Reagents	1. Conical Flask-50 mL		
Preparation of Reagents	1. NaoH-0.05M		
Sample Preparation	A) **Liquid or Strained honey**: If honey is free from granulation, mix thoroughly by stirring or shaking before weighing portion. If granulated, place closed sample container in water bath without submerging and heat at sample at 60°C for 30 min until liquefied. Occasional shaking is essential. cool the honey sample rapidly as soon it liquefies and mix thoroughly before taking test portion for determination. **Do not heat honey sample intended for diastase determination**. If foreign matter such as wax, sticks, bees, particles of comb etc. is present, heat honey to 40°C and filter through cheesecloth in hot water funnel before weighing, test portions for analysis. B) **Comb Honey**: Cut across top of comb, if sealed and separate completely from comb by straining through No. 40 sieve. When portions of comb or wax pass through sieve, heat product as in A and filter through cheese cloth. If honey is granulated in comb, heat sample until wax is liquefied and after this stir, cool, and remove wax.		
Method of analysis	1. Take 10 g of the sample in a suitable titration flask and dissolve it in 75 mL of carbon dioxide-free water. 2. Stir with magnetic stirrer; immerse electrodes of pH meter in solution, and record pH. 3. Titrate with 0.05M NaOH at rate of 5.0 mL/min. 4. Stop addition of NaOH at pH 8.5.		
Calculation with units of expression	Calculate as milliequivalent/kg Free acidity = (ml 0.05 M NaOH from burette – ml blank) x 50/g test portion		
Inference (Qualitative Analysis)	NA		
Reference	AOAC 962.19 AOAC (920.180)21st edition-2019		
Approved by	Scientific Panel on Methods of Sampling and Analysis		

FOOD SAFETY AND STANDARDS AUTHORITY OF INDIA Inspiring Trust, Assuring Safe & Nutritious Food Ministry of Health and Family Welfare, Government of India	**Determination of Hydroxy Methyl Furfural (HMF)**		
Method No.	FSSAI 04B.009:2023	**Revision No. & Date**	0.0
Scope	All types of Honey including Carvia Callosa and Honey dew		
Caution	1. Honey sample must be kept at moisture free place in air tight jar. 2. Mix the sample thoroughly before taking test portion for analysis. 3. Always wear gloves and mask while doing sample analysis.		
Principle	The determination of the Hydroxy Methyl Furfural (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 absorbance 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.		
Apparatus/Instruments	UV Spectrophotometer (284 and 336 nm wavelength)		
Materials and Reagents	1. Carrez solution I 2. Carrez solution II 3. Sodium bisulfite solution		
Preparation of Reagents	1. Carrez solution I- Dissolve 15 g Potassium ferrocyanide $K_4Fe(CN)_6.3H_2O$ and dilute to 100 mLwith water. 2. Carrez solution II- Dissolve 30 g Zinc acetate dehydrate Zn $(CH3COO)_2.2H_2O$ and dilute to 100 mL with water. 3. Sodium bisulfite solution- 0.20% Dissolve 0.20 g Sodium bisulfite ($NaHSO_3$) and dilute to 100 mL with water. Dilute 1 + 1 for dilution of reference solution if necessary. Prepare fresh solution daily.		
Sample Preparation	A) **Liquid or Strained honey**: If honey is free from granulation, mix thoroughly by stirring or shaking before weighing portion. If granulated, place closed sample container in water bath without submerging and heat at sample at 60°C for 30 min until liquefied. Occasional shaking is essential. cool the honey sample rapidly as soon it liquefies and mix thoroughly before taking test portion for determination. **Do not heat honey sample intended for diastase determination**. If foreign matter such as wax, sticks, bees, particles of comb etc. is present, heat honey to 40°C and filter through cheesecloth in hot water funnel before weighing, test portions for analysis. B) **Comb Honey**: Cut across top of comb, if sealed and separate completely from comb by straining through No. 40 sieve. When portions of comb or wax pass through sieve, heat product as in A and filter through cheese cloth. If honey is granulated in comb, heat sample until wax is liquefied and after this stir, cool, and remove wax.		
Method of analysis	1. Accurately weigh ca 5 g honey in small beaker and transfer with total of ca 25 mL H_2O to 50 ml volumetric flask. 2. Add 0.50 ml Careez solution I, mix and add 0.50 mL Careez solution II, mix and dilute to volume with water. Drop of alcohol may be added to suppress foam.		

	3. Filter through paper, discarding first 10 mL filtrate. 4. Pipet 5 mL filtrate into each of two 18 x 150 mm test tubes. 5. Add 5.0 mL H_2O to 1 tube (test solution) and 5.0 mL $NaHSO_3$ solution to other (reference). Mix well (Vortex mixer is useful) and determine A of test solution against reference at 284 and 336 nm in 1 cm cells. 6. If A is > 0.6, dilute test solution with H_2O and reference solution with 0.1% $NaHSO_3$ solution to same extent and correct A for dilution.
Calculation with units of expression	Hydroxymethyl furfural(HMF) = $\frac{(A284 - A336) \times 14.97 \times 5}{\text{g test sample}}$ mg 100 g honey Factor = 14.97 = (126/16830) (1000/10) (100/5) Where 126 = molecular weight HMF; 16830 = molar a of HMF at 284 nm; 1000 = mg/g; 10 =centiliters / L; 100 = g honey reported; 5 = nominal test portion weight.
Inference (Qualitative Analysis)	NA
Reference	AOAC official Methods 980.23 AOAC (920.180)21st edition-2019
Approved by	Scientific Panel on Methods of Sampling and Analysis

	Determination of Diastase Activity		
Method No.	FSSAI 04B.010:2023	**Revision No. & Date**	0.0
Scope	All types of Honey including Carvia Callosa and Honey dew		
Caution	1. Honey sample must be kept at moisture free place in air tight jar. 2. Mix the sample thoroughly before taking test portion for analysis. 3. Always wear gloves and mask while doing sample analysis. 4. Don't heat the sample before use.		
Principle	Diastase is an enzyme that is found naturally in honey and degrades over time, especially when exposed to heat. For determination of Diastase activity, Buffered soluble starch-honey solution is incubated and time required to reach specified end point is determined photometrically. Results are expressed as ml 1% starch hydrolyzed by enzyme in 1 g honey in 1 h.		
Apparatus/Instruments	1. **Reaction vessel**- Attach sealed side arm, 18 x 60 mm, to 18 x 175 mm test tube. Lower side of side arm is attached 100 mm from bottom of tube, making 45° angle with lower portion of tube. 2. **Visible Photo Spectrometer**- With 660 nm red filter or 600 nm interference filter and 1 cm cells.		
Materials and Reagents	1. Iodine stock solution 2. Iodine solution- 0.0007 M 3. Acetate buffer solution 4. Sodium chloride solution-0.5 M 5. Starch solution-2%		
Preparation of Reagents	1. **Iodine stock solution** - Dissolve 8.80 g resublimed I_2 in 30-40 mL H_2O containing 22.0 g KI, and dilute to 1 Lwith H_2O. 2. **Iodine solution** (0.0007 M) - Dissolve 20 g KI and 5.00 mL I_2 solution, (a) in H_2O and dilute to 500 mL. Prepare fresh every second day. 3. **Acetate buffer solution** (1.59 M) (pH 5.3) - Dissolve 87 g $NaCH_3COO.3H_2O$ in 400 mL H_2O, add ca 10.5 mL CH_3COOH in H_2O, and dilute to 500 mL. Adjust pH to 5.30 with $NaCH_3COO$ or CH_3COOH, if necessary. 4. **Sodium chloride solution (**0.5 M) - Dissolve 14.4 g NaCl in H_2O and dilute to 500 mL. 5. **Starch solution**- Weigh 2.000 g soluble starch (special for diastatic power determination) and mix with 90 mL H_2O in 250 ml Erlenmeyer. Rapidly bring to boiling point, swirling solution as much as possible. Reduce heat and boil gently 3 min, cover, and let cool to room temperature. Transfer to 100 ml volumetric flask and dilute to volume. Observe details closely to limit variation in absorbance (A) values of starch-I_2 blank.		
Sample Preparation	A) **Liquid or Strained honey**: If honey is free from granulation, mix thoroughly by stirring or shaking before weighing portion. If granulated, place closed sample container in water bath without submerging and heat at sample at 60°C for 30 min until liquefied. Occasional shaking is essential. cool the honey sample rapidly as soon it liquefies and mix thoroughly before taking test		

<table>
<tr><td></td><td>portion for determination. Do not heat honey sample intended for diastase determination. If foreign matter such as wax, sticks, bees, particles of comb etc. is present, heat honey to 40°C and filter through cheesecloth in hot water funnel before weighing, test portions for analysis.
B) Comb Honey: Cut across top of comb, if sealed and separate completely from comb by straining through No. 40 sieve. When portions of comb or wax pass through sieve, heat product as in A and filter through cheese cloth. If honey is granulated in comb, heat sample until wax is liquefied and after this stir, cool, and remove wax.</td></tr>
<tr><td>Method of analysis</td><td>1. Weigh 5 g test portion into 20 mL beaker, dissolve in 10-15 mL H_2O and 2.5 mL buffer solution, and transfer to 25 mL volumetric flask containing 1.5 mL NaCl solution.
2. Dilute to volume. (Solution must be buffered before addition to NaCl).
3. Pipet 5 mL starch solution into side arm of reaction tube and 10 mL test solution into bottom of tube, with care not to mix.
4. Place tube in water bath for 15 min at 40 ± 0.2 °C.
5. Then mix contents by tilting tube back and forth several times.
6. Start stopwatch. At 5 min, remove 1 mL aliquot with 1 mL serological pipette and add rapidly to 10.00 mL dilute I_2 solution in 50 mL graduate tube
7. Mix and dilute to previously determined volume and determine A in photometer.
8. Note time from mixing of starch and honey to addition of aliquot to I_2 as reaction time. (Place 1 mL pipette in reaction tube for reuse when later aliquots are taken.)
9. Continue taking 1 mL aliquots at intervals until A value of <0.235 is obtained.
10. Table given below shows absorbance values with corresponding end point times.
Absorbance values with corresponding end point
<table>
<tr><th>Absorbance</th><th>End Point</th></tr>
<tr><td>0.7</td><td>>25</td></tr>
<tr><td>0.65</td><td>20-25</td></tr>
<tr><td>0.6</td><td>15-18</td></tr>
<tr><td>0.55</td><td>11-13</td></tr>
<tr><td>0.5</td><td>9-10</td></tr>
<tr><td>0.45</td><td>7-8</td></tr>
</table></td></tr>
<tr><td>Calculation with units of expression</td><td>Plot A against time (min) on rectilinear paper; draw straight line through starting A and as many points as possible. From graph, determine time diluted 24eflon24n-I_2 mixture reaches A of 0.235.
Divide 300 by this time to obtain diastase number (DN).
[Notes: A 5 min reading is sufficient for approximating end point of test solutions of high DN (>35) if another value is taken soon enough to obtain A of ca 0.20. For accurate results, repeat determination, taking test solutions each min from start. With test solutions of low DN, another reading at 10 min will permit prediction of end point by plotting the data. No additional readings need be taken until within few minutes of end point. Only two such readings are needed. The 5 min value will not accurately predict low DN.]</td></tr>
</table>

Inference (Qualitative Analysis)	NA
Reference	AOAC Official Method 958.09 AOAC (920.180)21st edition-2019
Approved by	Scientific Panel on Methods of Sampling and Analysis

FOOD SAFETY AND STANDARDS AUTHORITY OF INDIA Inspiring Trust, Assuring Safe & Nutritious Food Ministry of Health and Family Welfare, Government of India	**Determination of Water insoluble matters**		
Method No.	FSSAI 04B.011:2023	**Revision No. & Date**	0.0
Scope	All types of Honey including Carvia Callosa and Honey dew		
Caution	1. Honey sample must be kept at moisture free place in air tight jar. 2. Mix the sample thoroughly before taking test portion for analysis. 3. Always wear gloves and mask while doing sample analysis.		
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.		
Apparatus/Instruments	1. Analytical balance, to 0.1mg. 2. Sintered glass crucible, pore size 15 to 40 microns. 3. Drying oven at 135 ± 10 ^{0}C.		
Materials and Reagents	NA		
Preparation of Reagents	NA		
Sample Preparation	Homogenize the sample before weighing.		
Method of analysis	1. Accurately weigh approximately 20 grams of honey and dissolve in about 200 ml of water at about 80 ^{0}C. Mix well. 2. 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. 3. Filter the sample solution through the crucible. 4. Wash carefully and extensively with warm water until free from sugars. 5. Check by adding to some filtrate in a test tube some 1% phloroglucinol in ethanol. 6. Mix and run a few drops of concentrated sulphuric acid down the sides of the tube. Sugars produce a colour at the interface. 7. Dry the crucible at 135°C for an hour, cool in the desiccator and weigh. 8. Dry again for 30 minute intervals until constant weight is obtained.		
Calculation with units of expression	% Insoluble Matter in g/100 m1= $\frac{M1}{M} \times 100$ where M1 = Mass of dried insoluble matter and M = Mass of honey taken		
Inference (Qualitative Analysis)	NA		
Reference	Harmonized Methods of the International Honey Commission (2009)		
Approved by	Scientific Panel on Methods of Sampling and Analysis		

FOOD SAFETY AND STANDARDS AUTHORITY OF INDIA Inspiring Trust, Assuring Safe & Nutritious Food Ministry of Health and Family Welfare, Government of India	**Determination of Pollen and Plant Elements**		
Method No.	FSSAI 04B.012:2023	**Revision No. & Date**	0.0
Scope	Honey		
Caution	1. Honey sample must be kept at moisture free place in air tight jar. 2. Mix the sample thoroughly before taking test portion for analysis, as heterogeneous distribution of pollen in honey		
Principle	Pollens present in honey are separated as sediment by centrifugation followed by staining with Basic Fuchsin. Stained pollens are then observed and counted under microscope using haemocytometer.		
Apparatus/Instruments	1. Microscope 10x10, 10x40 magnification capacity 2. Haemocytometer (1 mm square x 0.1 mm depth). 3. Centrifuge with rotor for 10,50 ml tubes 4. Weighing balance		
Materials and Reagents	1. Basic Fuchsin (0.5 percent alcoholic solution)		
Preparation of Reagents	1. 0.5 percent alcoholic solution: weigh 0.5gm of basic fuchsin in 95% ethanol		
Sample Preparation	1. Weigh accurately 10g of honey in a small clean beaker. Dissolve the honey in 50ml of distilled water. For honey rich in sediments, the quantity of honey may be reduced to 5g or 1g and dilution and calculation may suitably be altered. 2. Transfer this carefully to a 100mL measuring cylinder and fill the cylinder with distilled water upto 100mL mark. 3. Centrifuge 10mL of this stock solution in 15mL centrifuge tube at 3000 rev/min for 5 minutes. 4. Decant cautiously the supernatant liquid without disturbing the sediment, taking care to leave one millilitre of the liquid with the sediment in the tube. 5. Then, shake well the sediment and completely transfer to a collecting tube. Repeat centrifuging for all the stock solution of honey and sediments in the same collection tube. 6. To these sediments in the collection tube, add a drop of 0.5 percent alcoholic basic fuchsin solution and stir the sediment well. 7. Then centrifuge it and draw of the supernatant liquid and disperse the sediment in one millilitre of the solution.		
Method of analysis	1. Shake well the sediments and place a drop of this solution on the one millimeter squares on the haemocytometer and place a cover slip.		

	2. Count pollens present in one millimeter square at the magnification of 100 X. 3. Repeat this counting ten times and take 10 different counts with the dispersed sediment.
Calculation with units of expression	The average number of pollens counted over the haemocytometer is for the volume 0.1 mm (1 mm square X 0.1 mm depth). For this, calculate the pollens present in one millilitre, which is equivalent to their absolute number present in X g of honey taken for analysis. Express the results as the number of pollens in 1g of honey.
Inference (Qualitative Analysis)	Not Applicable
Reference	IS 4941:1994
Approved by	Scientific Panel on Methods of Sampling and Analysis

FOOD SAFETY AND STANDARDS AUTHORITY OF INDIA Inspiring Trust, Assuring Safe & Nutritious Food Ministry of Health and Family Welfare, Government of India	**Determination of Proline**		
Method No.	FSSAI 04B.013:2023	**Revision No. & Date**	0.0
Scope	All types of Honey including Carvia Callosa and Honey dew		
Caution	1. Honey sample must be kept at moisture free place in air tight jar. 2. Mix the sample thoroughly before taking test portion for analysis. 3. Always wear gloves and mask while doing sample analysis.		
Principle	Proline, predominant free amino acid of honey, reacts with acid ninhydrin solution to form colored compound. Interference from other amino acids is negligible, ≤5%.		
Apparatus/Instruments	1. Spectrophotometer 2. Reaction tubes- 18 x 130 mm borosilicate scew-cap tubes with 29eflon liners		
Materials and Reagents	1. Ninhydrin solution 2. L-(-)-Proline		
Preparation of Reagents	1. **Ninhydrin solution** (3%) - Dissolve 3.0 g Ninhydrin in 100 mL peroxide-free ethylene glycol monomethyl ether. Store solvent, not reagent, over Zn metal in amber bottle. 2. **L-(-)-Proline**- Dry in vaccum oven and store in desiccator. Prepare standard solutions as follows: a. Stock solution- 0.5 mg/mL H_2O. Dilute 25 mg Proline to 50 mL with H_2O and refrigerate it. b. Working solution- 50 µg/mL. Dilute to 10 mL stock solution to 100 mL with H_2O. Prepare working solution fresh daily.		
Sample Preparation	A) **Liquid or Strained honey**: If honey is free from granulation, mix thoroughly by stirring or shaking before weighing portion. If granulated, place closed sample container in water bath without submerging and heat at sample at 60°C for 30 min until liquefied. Occasional shaking is essential. cool the honey sample rapidly as soon it liquefies and mix thoroughly before taking test portion for determination. **Do not heat honey sample intended for diastase determination**. If foreign matter such as wax, sticks, bees, particles of comb etc. is present, heat honey to 40°C and filter through cheesecloth in hot water funnel before weighing, test portions for analysis. B) **Comb Honey**: Cut across top of comb, if sealed and separate completely from comb by straining through No. 40 sieve. When portions of comb or wax pass through sieve, heat product as in A and filter through cheese cloth. If honey is granulated in comb, heat sample until wax is liquefied and after this stir, cool, and remove wax.		
Method of analysis	1. Weigh 2.5 g honey into to 50 mL volumetric flask and makeup 50 mL volume with H_2O. 2. pipette 0.5 mL into beach of three reaction tubes, add 0.25 mL HCOOH and 1.00 mL Ninhydrin solution.		

	3. Cap tightly, shake well and place in boiling water for 15 min. 4. Cool 5 min in 22 °C water bath, remove cap, and pipette 5 mL aqueous Isopropanol (1 + 1) into each. 5. Mix well and determine A at 520 nm against blank of H_2O carried through method. 6. Read all tubes within 35 min of cooling. 7. Correct for color of honey by determining A of solution containing 0.5 mL prepared honey solution, 1.25 mL H_2O and 5.00 mL Isopropanol (1 + 1). 8. Subtract value from that of reacted test solution before calculating.
Calculation with units of expression	Prepare calibration curve as in determination, using Proline standard solution instead of honey. Absorbance (A) of 0.5 mL of solution of 50 µg proline/mL is ca 0.35 in 10 mm cell. Calculate Proline mg/100 g honey.
Inference (Qualitative Analysis)	NA
Reference	AOAC Official Method 979.20 AOAC (920.180)21st edition-2019
Approved by	Scientific Panel on Methods of Sampling and Analysis

FOOD SAFETY AND STANDARDS AUTHORITY OF INDIA Inspiring Trust, Assuring Safe & Nutritious Food Ministry of Health and Family Welfare, Government of India	**Determination of Electrical Conductivity**		
Method No.	FSSAI 04B.014:2023	**Revision No. & Date**	0.0
Scope	All types of Honey including Carvia Callosa and Honey dew		
Caution	1. Honey sample must be kept at moisture free place in air tight jar. 2. Mix the sample thoroughly before taking test portion for analysis. 3. Always wear gloves and mask while doing sample analysis.		
Principle	The determination of the electrical conductivity is based on the measurement of the electrical resistance, of which the electrical conductivity is the reciprocal. The electrical conductivity of a solution of 20 g dry matter of honey in 100 ml distilled water is measured using an electrical conductivity cell.		
Apparatus/Instruments	1. Conductivity meter, 2. Conductivity cell, platinized double electrode (immersion electrode). 3. Thermometer with divisions 0.10 ^{0}C. 4. Water bath, thermostatically controlled at a temperature of 20°C ± 0.5^{o}C. 5. Volumetric flasks, 100 mL and 1000 mL. 6. Beakers, tall form.		
Materials and Reagents	1. Potassium chloride solution		
Preparation of Reagents	1. **Potassium chloride solution** (0.1M) - Dissolve 7.4557 g of potassium chloride (KCl), dried at 130 °C, in freshly distilled water in a 1000 mL flask and fill to volume with distilled water. Prepare fresh on the day of use.		
Sample Preparation	A) **Liquid or Strained honey**: If honey is free from granulation, mix thoroughly by stirring or shaking before weighing portion. If granulated, place closed sample container in water bath without submerging and heat at sample at 60°C for 30 min until liquefied. Occasional shaking is essential. cool the honey sample rapidly as soon it liquefies and mix thoroughly before taking test portion for determination. **Do not heat honey sample intended for diastase determination**. If foreign matter such as wax, sticks, bees, particles of comb etc. is present, heat honey to 40°C and filter through cheesecloth in hot water funnel before weighing, test portions for analysis. B) **Comb Honey**: Cut across top of comb, if sealed and separate completely from comb by straining through No. 40 sieve. When portions of comb or wax pass through sieve, heat product as in A and filter through cheese cloth. If honey is granulated in comb, heat sample until wax is liquefied and after this stir, cool, and remove wax. Dissolve an amount of honey, equivalent to 20.0 g anhydrous honey, in distilled water. Transfer the solution quantitatively to a 100 mL volumetric flask and make up to volume with distilled water.		
Method of analysis	**Determination of the cell constant** If the cell constant of the conductivity cell is not known, proceed as follows: 1. Transfer 40 ml of the potassium chloride solution to a beaker. 2. Connect the conductivity cell to the conductivity meter, rinse the cell thoroughly with the potassium chloride solution . 3. Immerse the cell in the solution, together with a thermometer.		

	4. Read the electrical conductance of this solution in mS after the temperature has equilibrated to 20^0C. 5. *Calculate the cell constant K, using the following formula:* *K = 11.691 x 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 mean value of the electrical conductivity of freshly distilled water in mS.cm- and the electrical conductivity of a 0.1M potassium chloride solution, at 20 °C.* 6. Rinse the electrode thoroughly with distilled water after the determination of the cell constant. When not in use keep the electrode in distilled water in order to avoid ageing of the platinum electrode. ***Note:*** *If necessary, a 1 in 5 w/v dilution of a smaller amount of honey can be used.* 7. Pour 40 ml of the sample solution into a beaker and place the beaker in the thermostated water bath at 20 °C. 8. Rinse the conductivity cell thoroughly with the remaining part of the sample solution. 9. Immerse the conductivity cell in the sample solution. Read the conductance in mS after temperature equilibrium has been reached. ***Note:*** *a. Most conductivity meters are direct current. In order to avoid false results due to polarization effects, measurement time should as short 1as possible.* *b. If the determination is carried out at a different temperature, because of lack of thermostated cell, then a correction factor can be used for calculation of the value at 20 °C:* *i. For temperatures above 20 °C : subtract 3.2 % of the value per °C* *ii. For temperatures above 20 °C : subtract 3.2 % of the value per °C* *iii. For temperatures below 20 °C : add 3.2 % of the value per °C* *iv. For temperatures below 20 °C : add 3.2 % of the value per °C* c. Data from measurements corrected with the above factors values have not been validated in ring trials. d. However there were no significant differences between conductivity of 50 honeys, measured at 20 °C and at temperatures varying from 20 to 26 °C after applying the above correction factor (5).
Calculation with units of expression	Calculate the cell constant K, using the following formula: Calculate the electrical conductivity of the honey solution, using the following formula: $S_H = K . G$ Where: SH = electrical conductivity of the honey solution in mS.cm-1 K = cell constant in cm-1 G = conductance in mS Express the result to the nearest 0.01 mS.cm-1. G = the electrical conductance in mS, measured with the conductivity cell.

Inference (Qualitative Analysis)	NA
Reference	Harmonised Methods of the International Honey Commission (2009), AOAC (920.180)21st edition-2019
Approved by	Scientific Panel on Methods of Sampling and Analysis

	Determination of 2-Acetylfuran-3-Glucopyranoside (2-AFGP) as Marker for Rice Syrup		
Method No.	FSSAI 04B.015:2023	**Revision No. & Date**	0.0
Scope	All types of Honey		
Caution	1. Honey sample must be kept at moisture free place in air tight jar. 2. Mix the sample thoroughly before taking test portion for analysis. 3. Always wear gloves and mask while doing sample analysis.		
Principle	The method involves the dilution of honey with water; a clean-up through HLB cartridge and subsequent analysis by Liquid Chromatography- Mass Spectrometry (LC-MS/Ms).		
Apparatus/Instruments	1. High performance LC or Ultra-high-performance LC (UHPLC) system 2. Mass spectrometer: Triple-quadrupole mass spectrometer or equivalent MS/MS instrument. 3. Column: Agilent Eclipse plus C18 (100 mm x 4.6 mm, 3.5 µm)/Waters Acquity UPLC HSS PFP (100 x 2.1 mm, 1.8 µm) or equivalent.		
Materials and Reagents	1. Centrifuge Tubes (15 mL) 2. Analytical balance (Readability 0.0001 g) 3. Vortex mixer 4. Micro pipettes 20-200 µL and 100-1000 µL **Glassware & others:** 1. Injection vials 2. Volumetric flask Class A, 10 mL and 1 mL 3. Glass tubes 15 mL capacity 4. Hydrophilic syringe filters (0.22 µm) 5. Hydrophilic-Lipophilic-Balanced (HLB) water-wettable, reversed-phase sorbent cartridge or equivalent should be used for sample preparation. **Chemicals:** 1. Acetonitrile (MS Grade) 2. Methanol (MS Grade) 3. ASTM Type I Water/HPLC grade: Resistivity, min, 18.2 MΩ cm (at 25 °C) 4. Standard: 2-acetylfuran-3-glucopyranoside (AFGP)		
Preparation of Reagents	1. **Stock Solution**: Accurately weigh standard AFGP and add methanol as solvent make a stock solution of approximate 1.0 g/L (1000 mg/L which is same as 1 mg/mL) in a volumetric flask. 2. **Intermediate Standard Solution**: Prepare the intermediate standards of concentration of 10.0 mg/L (10000 µg/L) and 1.0 mg/L (1000 µg/L) by subsequent dilution with water.		

Concentration of stock standard (g/L)	**Vol. of stock solution (µL)**	**Vol. of water (µL)**	**Final conc. (g/L)**
1.0	100	900	0.1
0.1	100	900	0.01
0.01	100	900	0.001

3. **Working Standard (WS) Solution for calibration curve**: Prepare the working standards from the intermediate standard (0.001 g/L) by dilution with water as shown below.

Working standard concentration (µg/L (ppb)	**Volume of intermediate standard (µL)**	**Volume of water (µL)**	**Total volume (µL)**
100	100	900	1000
200	200	800	1000
300	300	700	1000
400	400	600	1000
800	800	100	1000
1000	100	0	1000
Note: If sample preparation is carried out using HLB cartridge the dilution must be carried out with methanol			

Sample Preparation

A. By dilution

1. Weigh 1 g ± 0.01 g of honey sample in a 15 ml centrifuge tube.
   Note (If the honey samples have particles centrifuge it at 5000 g for 5 minutes or pass through a nylon mesh (100-150 micron).
2. Add 1 ml water and shake vigoursly.
3. Dilute 1:5 if necessary.
4. Vortex the tubes for 5 minutes and rotospin for 5 minutes.
5. Centrifuge the tubes at 7000 x g for 5 min.
6. Collect upper clean extract and filter it through syringe filter (0.22 µm)
7. Use for LC-MS/MS

B. Using HLB cartridge

1. Take 1 g of honey sample in 15 mL centrifuge tube.
   (If the honey sample has particles centrifuge it at 5000 g for 5 min or pass through a nylon mesh (100-150 micron).
2. Add 5 mL ASTM Type I water and mix in a vortex for 3 min.
3. Make the volume up to 10 mL with water.
4. Take a 500 mg/6 cc HLB cartridge, condition it with methanol first then followed with water.
5. Pass the honey solution through the cartridge with constant speed and without applying any external pressure.
6. Elute the cartridge using 5.0 mL methanol.
7. Collect the elute in a clean tube.
8. Filter using 0.2 µm syringe filter prior to LC analysis.

Method of analysis

A. HPLC/UPLC configuration:

1. Set up the HPLC/UPLC system with the configuration shown below
   a. Column: C18 (100 mm x 4.6 mm, 3.5 µm)/(100 x 2.1 mm, 1.8 µm) or equivalent
   b. Injection volume: 10 µL
   c. Flow rate:0.5mL/min
   d. Elution: Gradient
   e. Solvent A: Water containing 0.1 % Formic acid
   f. Solvent B: Acetonitrile containing 0.1% Formic acid

II. Form Gradients by high-pressure mixing of two mobile phases, A and B, using the gradient programme shown below:

Gradient programme for HPLC/UPLC*		
Time (min)	Solvent A (%)	Solvent B (%)
Start	95	5
7	10	90
7.01	5	95
10	5	95
11	95	5
13	Stop	
*Gradient can be suitably modified and optimized to obtain best peak shape and resolution		

III. After verifying equilibration of the HPLC/UPLC system, inject the working standards followed by a reagent blank, control sample, and sample extracts. Injected working standards after the analysis of the last sample extract.

B. Mass spectrometer instrument settings:

Set up the mass spectrometer with instrument settings listed below

Gas temp. (°C)	300
Gas Flow (1/min)	10
Nebulizer (psi)	50
Sheath Gas Heater (°C)	300
Sheath Gas Flow (L/min)	10
Capillary (V)	3500
V Charging	500

Note: These settings are suitable for the 6460 triple-quadrupole (Agilent Technologies) mass spectrometer. Optimal tuning on alternative instrument will differ. Tune the instrument to obtain the precursor and product ions. Follow the manufacturer's instruction or alter conditions to obtain the best resolution of AFGP peaks.

Mass analysis parameters for AFGP							
AFGP ion	Precursor ion (m/z)	Product ion (m/z)	Dwell time (ms)	Fragmentor	#CE (V)	Cell Acceleration	Polarity
Analyte qualifier	311.07	185	100	162	9	7	Positive
Analyte quantifier	311.07	148.9	100	162	13	7	Positive
#CE: Collision Energy							

Peak Identification

a. Peak shape and response ratio of extracted ion chromatograms of sample should be similar to those obtained from calibration standard

b. The retention time of the AFPG in the extract should correspond to that of the calibration standard with a tolerance of ± 0.1 min.

c. Identification in MRM mode largely relies on the correct selection of ions.

d. Chromatographic peaks of different selected ions for the analyte must fully overlap.

e. Ion ratio from sample should be within ± 30% (relative) of average of calibration standards from same sequence

Calculation with units of expression	Acquire the chromatograms and prepare the calibration curve. Calculate the regression by plotting peak height response r for each working standard vs AFGP concentration. Carry out a regression analysis $R^2 = 0.999$

	Calculate the concentration of AFGP in the sample using the equation $y = mx + c$ Where, y = Area under the curve for AFGP in sample x = Concentration of Analyte m = Slope of the calibration curve c = value of y intercept The curve can also be directly taken from instrumental software. If the analyte concentration in sample is greater than the calibrated standards, the sample elute should be appropriately diluted and analyzed.
Inference (Qualitative Analysis)	If concentration of AFGP is < 1.0 mg/kg, results are reported as Absent/kg (MRPL 1mg/kg). If marker concentration is $\geq$ 1.0 mg/kg, results to be reported as Present/kg.
Reference	1. 2-Acetylfuran-3-Glucopyranoside as a Novel Marker For the Detection of Honey adulterated with Rice syrup. Xue Xiaofeng, Wang Qiang, Li Yi, Wu Liming, Chen Lanzhen, Zhao Jhing and Liu Fengmao. J. Agric. Food Chem., 2013, 61, 7488-7493p. 2. Rapid screening of multiclass syrup adulterants in honey by Ultra – Performance Liquid Chromatography/Quadrupole Time of Flight Mass Spectrometry, Du Bing, Wu Liming, Xue Xiaofeng, Chen Lanzhen, Zhao Jing and Cao Wei. J. Agric. Food Chem, 2015, 63(29), 6614-6623. 3. Guidance document on analytical quality control and method validation procedures for pesticide residues and analysis in food and feed; SANTE/11813/2017
Approved by	Scientific Panel on Methods of Sampling and Analysis

	Determination of C4 sugar, $\Delta\delta^{13}C_{protein\text{-}Honey}$ by EA-IRMS and $\Delta\delta^{13}C_{Fructose-Glucose}$, $\Delta\delta^{13}C_{max}$ Foreign Oligosaccharide by LC-IRMS		
Method No.	FSSAI 04B.016:2023	**Revision No. & Date**	0.0
Scope	Honey		
Caution	1. Honey sample must be kept at moisture free place in air tight jar. 2. Mix the sample thoroughly before taking test portion for analysis. 3. Always wear gloves and mask while doing sample analysis and reference material handling 4. Keep the eluent and reagent bottles under constant Helium purge to prevent CO2 contamination from ambient air. 5. Phosphoric acid and Sulphuric acid are highly corrosive. 6. Prepare the oxidation reagents fresh daily, store in dark brown bottle 7. Many of the routine gases for IRMS are hazardous. The laboratory should have an atmospheric monitoring system to warn of dangerous levels of gases.		
Principle	The method involves the determination of the relative isotopic ratios ($\delta^{13}C$) of 1) protein isolated from honey by EA-IRMS and 2) $\delta^{13}C$ values of every individual sugar present in honey within a single HPLC run by LC-IRMS. Isotopic ratios are measured relative to a working gas calibrated using internationally accepted standards and are reported using the delta notation (δ) and expressed as 'per mill (‰)'. The delta notation is defined as $\delta^{13}C(0/00)$ sample = [R(sample)/R(standard)-1] x 100 Where R represents the ratio $^{13}CO_2/^{12}CO_2$. The $^{13}C/^{12}C$ carbon isotope ratios reported as $\delta^{13}C$ values are related to Vienna Pee Dee Belemnite (VPDB) according to the AOAC Official Method 998.12. δ is the $^{13}C/^{12}C$ ratio of the sample related to the $^{13}C/^{12}C$ ratio of a reference material to ensure international compatibility of data sets. The unit of expression is, per mill (‰). The CO_2 produced from combustion of the protein fraction is isolated from the sample is analyzed to give $\delta^{13}C_{protein}‰$ by IRMS. The $\delta^{13}C‰$ values of fructose, glucose, disaccharides, and trisaccharide and any other oligosaccharides present in honey are determined by LC-IRMS. The sugars are separated by LC using a cation exchange column. All individual sugars eluting from LC column pass into the LC/IRMS interface. Here the carbon from organic samples in the mobile phase is converted into CO_2 by a wet chemical oxidation process using sodium peroxodisulfate either in the presence or absence of phosphoric acid. CO_2 and O_2 both diffuse through, which are subsequently dried in an online gas drying unit. The individual CO_2 peaks are subsequently admitted to the IRMS, which directly gives the $\delta^{13}C$ values for each individual sugar; $\delta^{13}C_{fru}‰$, $\delta^{13}C_{glu}‰$, $\delta^{13}C_{disaccharide}‰$, $\delta^{13}C_{trisaccharide}‰$ and $\delta^{13}C‰$ of any other oligosaccharides (see chromatograms below). The schematic of a typical LC-IRMS is shown below. The difference in the carbon isotope ratio between other $\delta^{13}C_{fru}‰$ and $\delta^{13}C_{glu}‰$ gives $\Delta\delta^{13}C_{fru\text{-}glu}‰$,		

<table>
<tr><td></td><td>$\delta^{13}C_{max}$ is the maximum difference observed between all possible isotopic ratios measured ($\Delta\delta^{13}C_{fru\text{-}disaccharides}$ / $\Delta\delta^{13}C_{fru\text{-}trisaccharides}$ / $\Delta\delta^{13}C_{fru\text{-}protein}$/ $\Delta\delta^{13}C_{fru\text{-}disaccharides}$ / $\Delta\delta^{13}C_{glu\text{-}trisaccharides}$ / $\Delta\delta^{13}C_{glu\text{-}protein}$ / $\Delta\delta^{13}C_{disaccharides\text{-}trisaccharides}$ / $\Delta\delta^{13}C_{disaccharides\text{-}protein}$ / $\Delta\delta^{13}C_{trisaccharides\text{-}protein}$)
The peak area (%) for foreign oligosaccharides is calculated from the areas appended in the LC chromatogram.</td></tr>
<tr><td>Apparatus/Instruments</td><td>1.An integrated EA-IRMS instrument equipped with an automated combustion system and mass spectrometer designed or modified for isotope ratio measurement at natural abundance
2.An integrated LC-IRMS comprising of HPLC/UPLC and in line oxidation reactor for aqueous oxidation of LC elute and a mass spectrometer designed or modified for isotope ratio measurement at natural abundance
3.LC comprises of a binary pump, autosampler, column oven (set at 80 °C), and cation exchange column (Ca^{2+}, 300 x 7.7 mm, 8 µm or equivalent)
4.Analytical microbalance: 0.0001 g
5.Micropipette: 10-100 µL, 20-200 µL and 100-1000 µL
6.Volumetric flasks: 10 mL Class A
7.Vortex mixer
8.Sonicator
9.Centrifuge (capable of 10,000 x g)
10.Water Bath (80 °C)
11.Convection oven
12.Centrifuge tubes (50 mL, 15 mL)
13.Spatula
14.Forceps (Blunt end and pointed curved end)
15.Tin capsules
16.Capsule holding tray
17.Nylon stocking material (100-150 mesh)
18.Syringe filters (0.45 µm and 0.22 µm)
19.Vaccum concentrator</td></tr>
<tr><td>Materials and Reagents</td><td>1. Stable Isotope reference standard

<table><tr><th>Certified Reference Standards</th><th>$\Delta^{13}C$ (‰)</th></tr><tr><td>Sucrose</td><td>-10.449</td></tr><tr><td>Casein</td><td>-26.98</td></tr><tr><td>NBS 22 Oil</td><td>-30.031</td></tr><tr><td>Beet Sugar</td><td>-26.027</td></tr><tr><td>Galactose</td><td>-21.415</td></tr><tr><td>Fructose</td><td>-10.985</td></tr><tr><td>Glucose</td><td>-10.97</td></tr><tr><td>Cane Sugar</td><td>-11.64</td></tr></table>
In-house standards for normalization and verified against above listed standards:
a. D-(-)-fructose ≥99% pure
b. D-(+)-glucose monohydrate ≥99.5% pure
c. D-(+)-sucrose ≥99% pure
d. D-(+)-maltose monohydrate ≥99% pure
e. D-(+)-raffinose pentahydrate ≥99% pure</td></tr>
</table>

	2. Ultra-pure water (Electrical Resistivity, Min.,18.18 MΩcm, at 25 °C) 3. Phosphoric acid (H_3PO_4) (purity ≥ 99%) 4. Sodium peroxodisulfate ($Na_2S_2O_8$, Sodium persulfate) (purum p. a. ≥ 99%) 5. Sodium tungstate dihydrate ($Na_2WO_4.2H_2O$) (puriss. p. a. ≥ 99%) 6. Sulfuric acid (p. a. 98%) 7. Tin capsules 8.CO_2 (working standard reference gas): 99.999% Pure 9.O_2 (flash combustion gas): > 99.999% Pure 10.Helium: 99.999% Pure
Preparation of Reagents	**Reagents for protein isolation** 1. 10% aqueous solution of Sodium tungstate: Dissolve 10 g of $Na_2WO_4.2H_2O$ in 100 mL of pure water. Prepare fresh daily 2. 0.335 M H_2SO_4: Dilute 1.88 mL concentrated H_2SO_4 to 100 mL with ultra-pure water **Chemical oxidation reagents** 1. 20% Sodium peroxodisulfate: Dissolve 200 g sodium peroxodisulfate in 1000 mL ultra-pure water in a brown glass bottle using an ultrasonic bath. Use a water-jet pump for vaccum degassing to remove all dissolved CO_2. 2. 1.5 M H_3PO_4 in water: Weigh 147.0 g of crystalline H_3PO_4. Dissolve in ~250 mL of ultra-pure water and make up to 1 L with water. **LC reagents** Ultra-pure water: (Electrical Resistivity, Min.,18.18 MΩcm, at 25 °C)
Sample Preparation	**1. EA-IRMS analysis** **A. Preparation of Standards for EA-IRMS** a. Weigh protein standard (Casein), approximately between 0.1-0.2mg, with the help of spatula in the tin capsule b. Fold the tin capsule with the help of the forceps in such a way so as to remove air. c. Gently fold it from all the sides and place the folded tin capsule in the carousel and start the sequence of operation following the manufacturer's instruction **B. Sample preparation for EA-IRMS:** a. Prepare in triplicate b. Strain honey through 100-150 mesh nylon stocking material to remove insoluble material. c. Add 4 mL H_2O to 10-12 g honey (in triplicate) in a 50 mL centrifuge tube and mix well to get a homogeneous solution d. Prepare fresh by mixing 2.0 mL 10% Na_2WO_4 solution and 2.0 mL 0.335 M H_2SO_4 in a small test tube. e. Add this mixture immediately to the diluted honey solution and mix well. f. Swirl the tube in ca 80 °C water bath until a visible flocculants (precipitate) forms with a clear supernatant. *Note: If no visible flocculants forms, or if supernatant remains cloudy, add 2 mL aliquots of 0.335 MH_2SO_4 with repeating heating between additions.* g. Fill tube with water, mix, centrifuge for 5 min at 6000 x g h. Decant supernatant. i. Repeat washing, mixing, and centrifuging steps nine times with ca 40 mL portions of water, thoroughly dispersing the pellet each time.

j. Dry protein at least for 3 h in ca 75 °C oven

k. Weigh approximately 0.1-0.2 mg isolated protein in tin capsules.

l. Gently fold the tin capsule with the help of forceps and place it

m. For $\delta\ ^{13}C_{honey}‰$, weigh filtered honey approximately 0.1-0.2 mg in tin capsules and follow step at (l).

Precautions:

a. Decant the supernatant immediately after centrifugation to avoid the mixing of pellet with the supernatant

b. Protein washing must be done very carefully to avoid any loss of pellet with the water

c. Fold the tin capsules gently to avid the leakage or loss of sample

d. Be careful during tin capsule folding to avoid air trapping.

C. Sample analysis on EA-IRMS

a. Placed the weighed casein standard, weighed protein and honey sample on the carousel of EA-IRMS for determining $\delta\ ^{13}C_{protein}$, $\delta\ ^{13}C_{honey}$

b. Operate the instrument as per manufacturer's instructions after calibration with CO_2 reference gas.

2. LC-IRMS analysis

A. Preparation of Standards for LC-IRMS

1. Prepare a solution of Fructose, Glucose, Sucrose and Raffinose containing 250mg/L of each in ultra-pure water.
2. Filter the solution through 0.22 µm syringe filter

B. Sample preparation for LC-IRMS analysis:

1. Strain honey through a 100-150 mesh size nylon stocking material
2. In triplicate accurately weigh about 200 mg sample in a 15 mL centrifuge tube. Mix well with 5 mL of Ulta-pure water.
3. Sonicate the mixture and make the volume up to 10 mL with water in a 10 mL volumetric flask.
4. Filter through 0.22 µm syringe filter into HPLC injection vials.

Note: Prepare sample solutions fresh everyday

C. Sample analysis on LC-IRMS

a. Introduce CO_2 reference gas pulse three times (20s each) at the beginning of each run.

b. The constant flow rate during this period gives the peaks a flattop appearance.

c. A level of CO_2 corresponding to 2-5 V (depending on the instrument) at m/z 44 is used to calibrate the system

d. Inject standard mixture (10µL) of fructose, glucose, disaccharide and trisaccharide. Repeat 10 times to obtain the mean and standard deviation for the $\delta^{13}C$ ‰ of individual sugars.

e. Inject Honey sample (10µL) in triplicate

f. The IRMS chromatogram provides details of the $\delta^{13}C‰$ of each of the sugars in the sample and the area under the curve of each of the resolve sugars.

g. The $\Delta\delta^{13}\ C_{fru\text{-}glu}$, $\Delta\delta^{13}\ C_{max}$ and foreign oligosaccharide content are calculated from the chromatogram data.

Method of analysis	**1. EA-IRMS conditions:** a. **EA conditions** (vario ISOTOPE cube, Elementar, UK) Temperature: Oxidation tube:950°C Reduction tube: 650°C Pressure:1300-1400mbar He flow: 230ml/min CO_2 flow: 230mL/min O_2 flow: 18mL/min b. **IRMS conditions (Isoprime)** Ion Source: CEI High Vacuum: 5e-6 Turbo speed: 100% TCD temperature:59°C Focus point:>0.5 Accelerating voltage:4000v Extraction voltage:76.00v Half plate differential (v): -121.00 Z-plate voltage (v): -53 Trap current (µA): 200 Electron volts (9ev): 75 Ion Repellor voltage(v): -9 Magnet current:4000 *The gas cylinders (associated valves etc) which supply working gases to the IRMS must be stored in a temperature-controlled environment.* 2. **LC-IRMS conditions:** **a. LC conditions** 1.Column: Ca^{2+} (300 x 7.7 mm, 8 µm) 2.Solvent: Ultra-pure water 3.Flow rate: 0.3 mL/min 4.Column Oven temperature: 80 °C 5.Injection volume: 10 µL **b. Interface for wet oxidation** (Isoprime Liquiface,Elementar,UK) 1.Reactor temperature: 95 °C 2.Oxidation reagent: 20% Sodium peroxodisulfate (Purge the solution with helium gas before use) 3.Flow rate: 60 µL/min *Note: Some instruments use 20% Sodium peroxodisulfate and 1.5 M H_3PO_4 for wet oxidation. Follow the manufacturer's instructions.* **c. IRMS Parameters (Isoprime IRMS):** Ion Source: CEI High Vaccum: $5e_{-6}$ Turbo Speed: 100% TCD temperature: 59 °C Focus point: >0.5 Accelerating Voltage: 4000v Extraction Voltage:76.00v Half Plate Differential(v): - 121.00 Z-Plate Voltage(v): -53.00 Trap current(µA): 200.00 Electron Volts (9ev): 75.00 Ion Repellor Voltage(v): -9.00 Magnet Current: 4000
Calculation with units of expression	1. $\Delta\delta^{13}C_{\text{protein-honey}}$: Subtract the $\delta^{13}C_{\text{protein}}$ (‰) value given in the chromatogram from the $\delta^{13}C_{\text{honey}}$ (‰) value. Report as $\boldsymbol{\Delta\delta^{13}C_{\text{protein-honey}}}$‰. 2. C4 Sugar (%): $$\frac{\delta^{13}C_{\text{Protein}} - \delta^{13}C_{\text{Honey}}}{\delta^{13}C_{\text{Protein}} - (-9.7)} \times 100$$

Where, -9.7 is the average $\delta^{13}C$ value for corn syrup, ‰. Report negative values from this calculation as 0%. Product is considered to contain significant C4 sugars (primarily corn or cane) only at or above of 7%.

3. $\Delta\boldsymbol{\delta^{13}C_{fru\text{-}flu}\%_0}$

Subtract the $\delta^{13}C_{Glu}$ (‰) value given in the chromatogram from the $\delta_{13}{}^{13}C_{Glu}$ (‰) value. Report as $\Delta\boldsymbol{\delta^{13}C_{fru\text{-}flu}}\%_0$.

4.$\Delta\boldsymbol{\delta^{13}C_{max}\%_0}$

Extract the $\delta^{13}C_{(}\%_0)_{values}$ of fructose, glucose, disaccharides and trisaccharides from the LC-IRMS profile.

Extract the $\delta^{13}C$ ‰ of protein from EA-IRMS profile and tabulate as shown

A $\delta^{13}C$ ‰	B $\delta^{13}C$ ‰	A-B $\Delta\delta^{13}C$ ‰
Fructose	Disaccharide	
Fructose	Trisaccharide	
Fructose	Protein	
Glucose	Disaccharide	
Glucose	Trisaccharide	
Glucose	Protein	
Disaccharide	Trisaccharide	
Disaacharide	Protein	
Trisaccharide	Protein	

The highest value observed in column three gives $\Delta\delta^{13}C_{max}\%_0$

5.**Foreign oligosaccharides (% peak area)**

Extract the area of individual peaks and calculate using the formula

Foreign oligosaccharide (area%)= Sum of the peak area of all peak(s) other than Fructose, Glucose, Disaccharides and Trisaccharides / Total peak area x 100

Inference (Qualitative Analysis)	NA
Reference	1. AOAC Official Method 998.12 C-4 Plants Sugar in Honey. Internal Standard Stable Carbon Isotope ration Method First Action 1998 2. Improved detection of honey adulteration by measuring differences between $^{13}C/^{12}C$ stable carbon isotope ratios of protein and sugar compounds with a combination of elemental analyzer – isotope ratio mass spectrometry and liquid chromatography – isotope ratio mass spectrometry ($\delta^{13}C$-EA/LCIRMS). Lutz Elfein, Kurt-Peter Raezke;Apidologie 2008, 39 (5), 574-587. 3.Liquid chromatography coupled to isotope ratio mass spectrometry: A new perspective on honey adulteration detection. Ana I. Cabanero, Jose L. Recio, Mercedes RupeaRez; J. Agric. Food Chem. 2006, 54, 9719-9727. 4.LC-IRMS: Authenticity control of honey using Thermo Scientific LZ IsoLink LC-IRMS. Andreas W.Hilkert, Michael krummen, Dieter Juchelka; Thermo application note 30024. 5.”Scientific support to the implementation of a Coordinated Control plan with a view to establishing the prevalence offraudulent practices in the marketing of

	honey" N° SANTE/2015/E3/JRC/S12.706828.E Aries, J. Burton,L. Carrasco, O. De Rudder, and A. Maquet. JRC Technical Report 2016, JRC104749, 38 p.
Approved by	Scientific Panel on Methods of Sampling and Analysis

B. BEES WAX

<table>
<tr><td>FOOD SAFETY AND STANDARDS AUTHORITY OF INDIA
Inspiring Trust, Assuring Safe & Nutritious Food
Ministry of Health and Family Welfare, Government of India</td><td colspan="3">Determination of Solubility</td></tr>
<tr><td>Method No.</td><td>FSSAI 04B.017:2023</td><td>Revision No. & Date</td><td>0.0</td></tr>
<tr><td>Scope</td><td colspan="3">Beeswax</td></tr>
<tr><td>Caution</td><td colspan="3">1. Sample must be kept at moisture free place in air tight jar.
2. Mix the sample thoroughly before taking test portion for analysis.
3. Always wear gloves and mask while doing sample analysis.
4. Keep the sample at dry and cool place.</td></tr>
<tr><td>Principle</td><td colspan="3">Solubility of bees wax is determined by adding known amount of sample into known volume of various solvent i.e. Alcohol, Ether and Water.</td></tr>
<tr><td>Apparatus/Instruments</td><td colspan="3">Conical Flask</td></tr>
<tr><td>Materials and Reagents</td><td colspan="3">1. Ethanol
2. Ether
3. Water</td></tr>
<tr><td>Preparation of Reagents</td><td colspan="3">NA</td></tr>
<tr><td>Sample Preparation</td><td colspan="3">Melt the sample, if necessary, and filter it through a dry filter paper to remove any traces of moisture.</td></tr>
<tr><td>Method of analysis</td><td colspan="3">A. Procedure: Transfer a known amount of the sample into a flask containing known amount of the specified solvent, shake for not less than 30 sec and not more than 5 min.

<table>
<tr><th>Descriptive term</th><th>Parts of solvent required for 1 part of solute</th></tr>
<tr><td>Very soluble</td><td>Less than 1</td></tr>
<tr><td>Freely soluble</td><td>From 1 to Less than 10</td></tr>
<tr><td>Soluble</td><td>From 10 to Less than 30</td></tr>
<tr><td>Sparingly soluble</td><td>From 30 to Less than 100</td></tr>
<tr><td>Slightly soluble</td><td>From 100 to Less than 1,000</td></tr>
<tr><td>Very slightly soluble</td><td>From 1,000 to Less than 10,000</td></tr>
<tr><td>Practically insoluble or in soluble</td><td>More than 10,000</td></tr>
</table>

B. Solubility in Ethanol:
1. Transfer a 1 mL sample into a calibrated 10-mL glass-stoppered cylinder graduated in 0.1-mL subdivisions
2. Add slowly, in small portions, ethanol, the concentration and quantity of which are specified in the monograph.
3. Maintain the temperature at 20°C.
4. A clear solution, free from foreign matter should be obtained.</td></tr>
<tr><td>Calculation with units of expression</td><td colspan="3">NA</td></tr>
<tr><td>Inference (Qualitative Analysis)</td><td colspan="3">NA</td></tr>
<tr><td>Reference</td><td colspan="3">JECFA INS 901 and JECFA combined compendium of food additives specification volume 4</td></tr>
<tr><td>Approved by</td><td colspan="3">Scientific Panel on Methods of Sampling and Analysis</td></tr>
</table>

	Determination of Melting Point		
Method No.	FSSAI 04B.018:2023	**Revision No. & Date**	0.0
Scope	Bees wax		
Caution	1. Sample must be kept at moisture free place in air tight jar. 2. Mix the sample thoroughly before taking test portion for analysis. 3. Always wear gloves and mask while doing sample analysis. 4. Keep the sample at dry and cool place.		
Principle	Bees wax softens or become sufficiently fluid to slip or clear at given temperature which is determined by capillary-slip method.		
Apparatus/Instruments	1. Thermometer of a suitable type, with an accuracy of 0.1 °C and graduated at every 0.1 °C. 2. Test Tube- with a centrally bored cork to take the thermometer. The cork shall have a slit so as to permit circulation of air. 3. Water Bath, with the thermometer.		
Materials and Reagents	NA		
Preparation of Reagents	NA		
Sample Preparation	Before determining the melting range of a substance, the sample should be dried under the conditions specified for Loss on Drying in the individual monograph. If a temperature is not specified in the monograph, the sample should be dried for 24 h in a desiccator.		
Method of analysis	1. Transfer a quantity of the dried powder to a dry capillary-tube about 10 cm long and sealed at one end (thickness of the wall, 0.10-0.15 mm; i.d. 0.9-1.1 mm) and pack the powder by tapping the tube on a hard surface so as to form a tightly-packed column 2-4 mm in height. 2. Attach the capillary-tube and its contents to a standard thermometer so that the closed end is at the level of the middle of the bulb, and heat in a suitable apparatus containing an appropriate liquid (liquid paraffin or silicone oil) and fitted with a stirring device and an auxiliary thermometer. 3. Regulate the rise in temperature during the first period to 3° per min. 4. When the temperature has risen to 5° below the lowest figure of the range for the substance being tested, heat more slowly: if no other directions are given, the rate of rise in temperature should be 1-2° per min, Unless otherwise directed. 5. Read the temperature at which the substance is observed to form droplets against the side of the tube and the temperature at which it is completely melted, as indicated by the formation of a definitive meniscus.		

Calculation with units of expression	Before starting the determination of the melting range, adjust the auxiliary thermometer so that the bulb touches the standard thermometer at a point midway between the graduation for the expected melting range and the surface of the heating material. When the substance has melted, read the temperature on the auxiliary thermometer. Calculate the correction to be added to the temperature reading of the standard thermometer from the following formula: $0.00015\ N(T - t)$ in which T is the temperature reading of the standard thermometer, t is the temperature reading of the auxiliary thermometer and N is the number of degrees of the scale of the standard thermometer between the surface of the heating material and the level of the mercury. The statement "melting range, a^o - b^o" means that the corrected temperature at which the material is observed to form droplets must be at least a^o, and that the material must be completely melted at the corrected temperature b^o.
Inference (Qualitative Analysis)	NA
Reference	JECFA INS 901 and JECFA combined compendium of food additives specification volume 4
Approved by	Scientific Panel on Methods of Sampling and Analysis

FOOD SAFETY AND STANDARDS AUTHORITY OF INDIA Inspiring Trust, Assuring Safe & Nutritious Food Ministry of Health and Family Welfare, Government of India	**Determination of Acid Value**		
Method No.	FSSAI 04B.019:2023	**Revision No. & Date**	0.0
Scope	Bees wax		
Caution	1. Sample must be kept at moisture free place in air tight jar. 2. Mix the sample thoroughly before taking test portion for analysis. 3. Always wear gloves and mask while doing sample analysis. 4. Keep the sample at dry and cool place.		
Principle	Acid Value is the number of milligrams of potassium hydroxide (KOH) necessary to neutralize the fatty acids in 1 gram of sample. Acid value is determined by directly titrating the alcoholic solution of test sample with aqueous potassium hydroxide solution.		
Apparatus/Instruments	1.Burette 2. Erlenmeyer flask		
Materials and Reagents	1) Neutral ethanol 2) Neutralized diethyl ether/ethanol or petroleum spirit/ethanol 3) Phenolphthalein 4) 0.5 N KOH		
Preparation of Reagents	1) Neutral ethanol-95% 2) Neutralized diethyl ether/ethanol or petroleum spirit/ethanol 3) Phenolphthalein-Dissolve One gram of phenolphthalein indicator in 100 mL of ethyl alcohol		
Sample Preparation	Melt the sample, if necessary, and filter it through a dry filter paper to remove any traces of moisture.		
Method of analysis	1. Weigh accurately about 5 g of sample into a 500- mL Erlenmeyer flask. 2. Add 75-100 mL of hot neutral ethanol. 3. Heat and agitate the sample solution. 4. For some samples, it may be necessary to use as the solvent a 1:1 mixture of neutralized diethyl ether/ethanol or petroleum spirit/ethanol. 5. Add 0.5 mL of phenolphthalein and titrate immediately, while shaking, with 0.5 N KOH until the pink colour persists for at least 30 sec. 6. For acidity less than 2% by weight, 0.1 N KOH should be used for the titration. 7. For acidity less than 0.2% by weight, it is necessary, in addition, to first neutralize the carbon dioxide in the reaction vessel.		
Calculation with units of expression	Acid value = (56.1 x T x N) / W Where T is the titre (ml); N is the normality of potassium hydroxide solution; and W is the weight of sample (g).		
Inference (Qualitative Analysis)	NA		

Reference	Food Chemical Codex 2016
Approved by	Scientific Panel on Methods of Sampling and Analysis

FOOD SAFETY AND STANDARDS AUTHORITY OF INDIA Inspiring Trust, Assuring Safe & Nutritious Food Ministry of Health and Family Welfare, Government of India	**Determination of Peroxide Value**		
Method No.	FSSAI 04B.020:2023	**Revision No. & Date**	0.0
Scope	Bees wax		
Caution	1. Sample must be kept at moisture free place in air tight jar. 2. Mix the sample thoroughly before taking test portion for analysis. 3. Always wear gloves and mask while doing sample analysis. 4. Keep the sample at dry and cool place.		
Principle	The peroxide value is a measure of the peroxides contained in a sample of wax, expressed as milli-equivalents of peroxide per 1000 grams.		
Apparatus/Instruments	1. Burette (50 mL) 2. Conical Flask(250 mL)		
Materials and Reagents	1. Chloroform 2. Acetic Acid 3. Potassium Iodide Solution 4. 0.01N sodium thiosulfate 5. Starch-1%		
Preparation of Reagents	1. Potassium Iodide Solution-Saturated: Prepare saturated solution of potassium iodide in boiled distilled water and store in dark. 2. Acetic Acid- Chloroform solution: Mix three parts by volume of glacial acetic acid with 2 parts by volume of chloroform.		
Sample Preparation	Melt the sample, if necessary, and filter it through a dry filter paper to remove any traces of moisture.		
Method of analysis	1. Weigh accurately 5 g of the sample into a 200 mL conical flask. 2. Add 30 mL of a 2:3 solution of chloroform and acetic acid and close the flask with a stopper. 3. Heat with warm water and swirl to dissolve the sample. 4. Cool to room temperature and add 0.5 mL of saturated potassium iodide solution. 5. Close the flask with the stopper and shake vigorously for 60 ± 5 sec. Add 30 mL of water and titrate immediately with 0.01 N sodium thiosulfate using starch as indicator. 6. Carry out a blank determination.		
Calculation with units of expression	Peroxide value = (a-b) x N x 1000/W where a = Volume (ml) of sodium thiosulfate used for the sample b = Volume (ml) of sodium thiosulfate used for the blank N = Normality of the sodium thiosulfate W = Weight of sample (g)		
Inference (Qualitative Analysis)	NA		

Reference	JECFA INS 901 and JECFA combined compendium of food additives specification volume 4, IS:548(Part 1)-1964
Approved by	Scientific Panel on Methods of Sampling and Analysis

FOOD SAFETY AND STANDARDS AUTHORITY OF INDIA Inspiring Trust, Assuring Safe & Nutritious Food Ministry of Health and Family Welfare, Government of India	**Determination of Saponification Value**		
Method No.	FSSAI 04B.021:2023	**Revision No. & Date**	0.0
Scope	This method is applicable for Bees wax		
Caution	1. Sample must be kept at moisture free place in air tight jar. 2. Mix the sample thoroughly before taking test portion for analysis. 3. Always wear gloves and mask while doing sample analysis. 4. Keep the sample at dry and cool place.		
Principle	The test sample is saponified by refluxing with a excess of alcohol potassium hydroxide solution. The alkali consumed for saponification is determined by titrating the excess alkali with standard hydrochloric acid.		
Apparatus/Instruments	1. Conical Flask-250 to 300 mL capacity made of alkali-resistant glass. 2. Reflux Air Condenser-at least 65 cm long.		
Materials and Reagents	1. Methyl Ethyl Ketone 2. Rectified Spirit 3. Alcoholic potassium Hydroxide solution 4. Phenolphthalein Indicator Solution 5. Standard Hydrochloric Acid		
Preparation of Reagents	1. Methyl Ethyl Ketone- This shall be stored in dark 2. Rectified Spirit-Neutral to phenolphthalein indicator 3. Alcoholic potassium Hydroxide solution- Dissolve 30 g of potassium hydroxide in rectified spirit and make up to 1 litre. Allow to settle overnight in a dark place, decant the clear liquid and keep in a bottle closed tight with cork or rubber stopper. 4. Phenolphthalein Indicator Solution- Dissolve 0.1 g of phenolphthalein in 60 mL of rectified spirit and dilute with water to 100 mL 5. Standard Hydrochloric Acid-0.5 N		
Sample Preparation	Melt the sample, if necessary, and filter it through a dry filter paper to remove any traces of moisture.		
Method of analysis	1. weigh accurately into a 250 mL flask a sample of such size (usually about 4-5 g) that the titration of the sample solution after saponification will require between 45 and 55% of the volume of 0.5 N hydrochloric acid required for the blank. 2. Add 50.0 ml of ethanolic potassium hydroxide from a pipette and allow the pipette to drain for a definite period of time. 3. Prepare and conduct blank determinations simultaneously with the sample and similar in all respects. 4. Connect an air condenser to each flask and boil gently but steadily, with occasional mixing, until the sample is completely saponified. (This usually Requires about 1 h for normal samples).		

	5. After the flasks and condensers have cooled somewhat but not sufficiently for the contents to gel, wash down the inside of the condensers with a few mL of distilled water. 6. Disconnect the condensers, add about 1 mLof phenolphthalein to reach flask, and titrate with 0.5 N hydrochloric acid until the pink colour has just disappeared.
Calculation with units of expression	Saponification value = [56.1 x N (A - B)] / W Where A is mL of HCl required for the titration of the blank; B is mL of HCl required for the titration of the sample; W is the weight of sample in g; and N is normality of the HCl.
Inference (Qualitative Analysis)	NA
Reference	Food Chemical Codex 2016
Approved by	Scientific Panel on Methods of Sampling and Analysis

	Determination of Carnauba Wax		
Method No.	FSSAI 04B.022:2023	**Revision No. & Date**	0.0
Scope	Bees wax		
Caution	1. Sample must be kept at moisture free place in air tight jar. 2. Mix the sample thoroughly before taking test portion for analysis. 3. Always wear gloves and mask while doing sample analysis. 4. Keep the sample at dry and cool place.		
Principle	When the sample is dissolved in n-butanol and boiling followed by cooling is done, loose mass of fine needle-like crystals separates from clear mother liquor. Further examination is done under microscope.		
Apparatus/Instruments	1. Microscope		
Materials and Reagents	2. n-butanol		
Preparation of Reagents	NA.		
Sample Preparation	NA.		
Method of analysis	1. Transfer 100 mg of the sample into a test tube, and add 20 mL of n-butanol. 2. Immerse the test tube in boiling water, and shake the mixture gently until the sample dissolves completely. 3. Transfer the test tube to a beaker of water at 60 °C, and allow the water to cool to room temperature. 4. A loose mass of fine, needle-like crystals separates from clear mother liquor. 5. Under the microscope, the crystals appear as loose needles or stellate clusters, and no amorphous masses are observed, indicating the absence of carnauba wax.		
Calculation with units of expression	NA		
Inference (Qualitative Analysis)	NA		
Reference	JECFA INS 901		
Approved by	Scientific Panel on Methods of Sampling and Analysis		

	Determination of Ceresins, Paraffins and other waxes		
Method No.	FSSAI 04B.023:2023	**Revision No. & Date**	0.0
Scope	Bees wax		
Caution	1. Sample must be kept at moisture free place in air tight jar. 2. Mix the sample thoroughly before taking test portion for analysis. 3. Always wear gloves and mask while doing sample analysis. 4. Keep the sample at dry and cool place.		
Principle	The sample is refluxed with a known excess of alcohol potassium hydroxide solution, which lead to solution become clear at given temperature. Any kind of precipitation indicate the presence of Ceresins, paraffins and other waxes		
Apparatus/Instruments	1. Round-bottomed flask 2. Reflux condenser 3. Thermometer		
Materials and Reagents	1. Alcoholic Potassium hydroxide 2. Aldehyde-free ethanol		
Preparation of Reagents	1. Alcoholic Potassium hydroxide- Approximately 0.5 N, prepared by dissolving potassium hydroxide in rectified spirit. 2. Aldehyde-free ethanol- To 125 mL alcohol contained in 1000 mL flask, add 375 mL of dinitrophenylhydrazine solution, heat on a water bath under a reflux condenser for twenty-four hours, remove the alcohol by distillation, dilute to 100 ml with a 2 percent v/v solution of sulphuric acid, and set aside for 24 hours.		
Sample Preparation	NA		
Method of analysis	1. Transfer 3.0 g of the sample to a 100 mL round-bottomed flask. 2. Add 30 mL of a 4% w/v solution of potassium hydroxide in aldehyde-free ethanol and boil gently under a reflux condenser for 2 h. 3. Remove the condenser and immediately insert a thermometer. Place the flask in water at 80 °C and allow to cool, swirling the solution continuously. 4. Observe any kind of precipitation before the temperature reaches 65 °C, although the solution may be opalescent.		
Calculation with units of expression	NA		
Inference (Qualitative Analysis)	Any kind of precipitation indicate the presence of Ceresins, paraffins and certain other waxes		
Reference	JECFA INS 901, IS 4028:1992		
Approved by	Scientific Panel on Methods of Sampling and Analysis		

	Determination of Fats, Japan wax, Rosin and Soap		
Method No.	FSSAI 04B.024:2023	**Revision No. & Date**	0.0
Scope	Bees wax		
Caution	1. Sample must be kept at moisture free place in air tight jar. 2. Mix the sample thoroughly before taking test portion for analysis. 3. Always wear gloves and mask while doing sample analysis. 4. Keep the sample at dry and cool place.		
Principle	When Sample is boiled in solution of sodium hydroxide, followed by cooling, filtration and acidification with hydrochloric acid. Any kind of precipitation indicates the presence of Fats, Japan wax, rosin and soap.		
Apparatus/Instruments	NA		
Materials and Reagents	1. Sodium hydroxide Solution 2. Dilute Hydrochloric Acid		
Preparation of Reagents	1. Sodium hydroxide Solution- 10 percent (m/v). 2. Dilute Hydrochloric Acid – approximately 4 N.		
Sample Preparation	NA		
Method of analysis	1. Boil 1 g of the sample for 30 min with 35 ml of a 1 in 7 solution of sodium hydroxide, maintaining the volume by the occasional addition of water, and cool the mixture. 2. The wax separates and the liquid remains clear. 3. Filter the cold mixture and acidify the filtrate with hydrochloric acid. 4. There should be no precipitation.		
Calculation with units of expression	NA		
Inference (Qualitative Analysis)	Any kind of precipitation indicate the presence of Fats, Japan wax, rosin and soap		
Reference	JECFA INS 901, IS 4028-1992		
Approved by	Scientific Panel on Methods of Sampling and Analysis		

	Determination of Glycerol and other polyols		
Method No.	FSSAI 04B.025:2023	**Revision No. & Date**	0.0
Scope	Bees wax		
Caution	1. Sample must be kept at moisture free place in air tight jar. 2. Mix the sample thoroughly before taking test portion for analysis. 3. Always wear gloves and mask while doing sample analysis. 4. Keep the sample at dry and cool place.		
Principle	After 30 minutes of refluxing with ethanolic potassium hydroxide, the acidic filtrate is combined with decolorized fuchsin chemical to produce a blue colour.		
Apparatus/Instruments	1. Round-bottom flask 2.Beaker		
Materials and Reagents	1. Ethanolic potassium hydroxide 2. Sulfuric acid 3.Sodium periodate 4. Decolourized fuchsin solution		
Preparation of Reagents	1) Decolourized fuchsin solution- Dissolve 0.1 g of basic fuchsin in 60 mL of water. Add a solution of 1 g of anhydrous sodium sulfite (Reagent grade) in 10 mL of water. Slowly and with continuous shaking of the solution add 2 mL of hydrochloric acid. Dilute to 100 mL with water. Allow to stand protected from light for at least 12 h, decolourize with activated charcoal and filter. If the solution becomes cloudy, filter before use. If on standing the solution becomes violet, decolourize again by adding activated charcoal. Store protected from light.		
Sample Preparation	NA		
Method of analysis	1. To 0.20 g of the sample in a round-bottom flask, add 10 mL of ethanolic potassium hydroxide TS, attach a reflux condenser to the flask and heat in a water bath for 30 min. 2. Add 50 mL of dilute sulfuric acid cool and filter. 3. Rinse the flask and filter with dilute sulfuric acid TS. 4. Combine the filtrate and washings and dilute to 100.0 mL with dilute sulfuric acid TS. 5. Place 1.0 mL of the solution in a tube, add 0.5 mL of a 1.07 % (w/v) solution of sodium periodate. 6. Mix and allow standing for 5 min. 7. Add 1.0 mL of decolorized fuchsin solution and mix. Any precipitate disappears. 8. Place the tube in a beaker containing water at 40 °C. 9. Allow to cool while observing for 10 to 15 min. Any bluish-violet colour in the solution is not more intense than a standard prepared at the same time in the same manner using 1.0 mL of a 0.001 % (w/v) solution of glycerol in dilute sulfuric acid.		

	10. Bluish-Violet color should not be more intensive than a standard.
Calculation with units of expression	NA.
Inference (Qualitative Analysis)	More intensive Bluish-Violet color than standard indicate the presence of Glycerol and other polyols.
Reference	JECFA INS 901
Approved by	Scientific Panel on Methods of Sampling and Analysis

	Determination of Ash		
Method No.	FSSAI 04B.026:2023	**Revision No. & Date**	0.0
Scope	Bees wax		
Caution	1. Sample must be kept at moisture free place in air tight jar. 2. Mix the sample thoroughly before taking test portion for analysis. 3. Always wear gloves and mask while doing sample analysis. 4. Keep the sample at dry and cool place.		
Principle	The sample ashed at a temperature 650 °c for 1 hr and the residue weighed and calculated as ash content.		
Apparatus/Instruments	1. Platinum Dish- having a capacity of 100 mL 2. Muffle Furnace 3.Dessicator		
Materials and Reagents	NA		
Preparation of Reagents	NA		
Sample Preparation	NA		
Method of analysis	1. Heat the platinum dish to redness, cool to room temperature in a desiccator and weigh. 2. Take about 50 g of the material in a watch glass and weigh accurately. 3. Transfer about three quarters of this quantity to the platinum dish and heat on a Bunsen burner so that the material burns gently at the surface. 4. When about half of the material is burnt away, stop heating, cool and add the remainder of the material. 5. Weigh the watch glass again and find, by difference, the exact mass of sample transferred to the platinum dish. 6. Heat again till the material is completely charred. 7. Incinerate in a muffle furnace at 550 °C to 650 °C for 1 h. 8. Cool to room temperature in a desiccator and weigh. 9. Repeat incineration, cooling and weighing until the difference between two successive weighing is less than one milligram.		
Calculation with units of expression	Ash, percent by mass $= \frac{M_2 \times 100}{M_1}$ Where M_2 = mass in g of the ash; and M_1 = mass in g of the material taken for the test		
Inference (Qualitative Analysis)	NA		

Reference	IS 4028:1992
Approved by	Scientific Panel on Methods of Sampling and Analysis

FOOD SAFETY AND STANDARDS AUTHORITY OF INDIA Inspiring Trust, Assuring Safe & Nutritious Food Ministry of Health and Family Welfare, Government of India	**Determination of Total Volatile matter**		
Method No.	FSSAI 04B.027:2023	**Revision No. & Date**	0.0
Scope	Bees wax		
Caution	1. Sample must be kept at moisture free place in air tight jar. 2. Mix the sample thoroughly before taking test portion for analysis. 3. Always wear gloves and mask while doing sample analysis. 4. Keep the sample at dry and cool place.		
Principle	Total volatile matter is determined by weighing the sample before and after drying and determining the difference.		
Apparatus/Instruments	1. Oven 2. Desiccator		
Materials and Reagents	NA		
Preparation of Reagent	NA		
Sample Preparation	NA		
Method of analysis	1. Weigh accurately about 10 g of the material in a suitable dish, previously dried and weighed, and place it in an oven maintained at 105 ± 2 °C for 6 h. 2. Cool the dish in a desiccator and weigh with the lid on. 3. Heat the dish again in the oven for 30 min. 4. Repeat the process until the loss in mass between two successive weighing is less than one milligram. Record the constant mass obtained.		
Calculation with units of expression	Total volatile matter at 105 °C, percent by mass = $\frac{100\,(M_1 - M_2)}{M_1 - M_3}$ Where M_1 = mass in g of the dish with the material before heating; M_2 = mass in g of the dish after heating; and M_3 = mass in g of the empty dish.		
Inference (Qualitative Analysis)	NA		
Reference	IS 4028-1992		
Approved by	Scientific Panel on Methods of Sampling and Analysis		

C. Royal jelly

FOOD SAFETY AND STANDARDS AUTHORITY OF INDIA Inspiring Trust, Assuring Safe & Nutritious Food Ministry of Health and Family Welfare, Government of India	**Determination of Moisture (Vacuum Oven Drying Method : Reference Method)**		
Method No.	FSSAI 04B.028:2023	**Revision No. & Date**	0.0
Scope	Royal Jelly		
Caution	Properly mix the sample before analysis and it should be free from bubbles.		
Principle	Royal jelly sample is heated in a vacuum oven under controlled conditions of pressure and temperature to remove moisture by passing dry air. Sample is weighed before and after drying to estimate moisture.		
Apparatus/Instruments	1. Vacuum drying oven 2. Weighing dish, (height 25 mm to ~30 mm, of diameter 35 mm to 50 mm). 3. Analytical Balance, (weighing to the nearest 0, 0001 g). 4. Desiccator		
Materials and Reagents	Desiccants		
Preparation of Reagents	NA		
Sample Preparation	Homogenize the sample before weighing		
Method of analysis	1. Weigh approximately 0.5 g of royal jelly sample in the weighing dish which is dried to constant weight, spread the sample evenly weigh accurately. 2. Put the dish with sample in the vacuum drying oven . 3. Dry for 4 h at 75 °C under the pressure between 0,000 Mpa and 0,005 Mpa. 4. Take out the weighing dish and put it in the desiccators. 5. Weigh after it has been cooled for 30 min. 6. Re dry for 2 h and repeat the process until the weight difference between two consecutive times is no more than 2 mg, until a constant weight is achieved.		
Calculation with units of expression	Moisture (%) (by weight) $= \frac{W_1 - W_2}{W1 - W} \times 100$ Where, W = Weight in g, of Aluminium dish. W_1 = Weight in g, of Aluminium dish + sample before drying. W_2 = Weight in g, of Aluminium dish + dried sample until constant weight		
Inference (Qualitative Analysis)	NA		

Reference	IS/ISO 12824:2016
Approved by	Scientific Panel on Methods of Sampling and Analysis

FOOD SAFETY AND STANDARDS AUTHORITY OF INDIA Inspiring Trust, Assuring Safe & Nutritious Food Ministry of Health and Family Welfare, Government of India	**Determination Moisture (Karl Fisher Method)**		
Method No.	FSSAI 04B.029:2023	**Revision No. & Date**	0.0
Scope	Royal Jelly		
Caution	Properly mix the sample before analysis and it should be free from bubbles.		
Principle	The Karl Fisher reaction is based upon the oxidation of sulfur dioxide by iodine with the consumption of water in a buffered solution. Water reacts with iodine and sulphur dioxide to form sulphur trioxide and hydrogen iodide. An endpoint is reached when all the water is consumed. The water content is then calculated from the amount of reagent added.		
Apparatus/Instruments	**Karl Fisher** 1. Karl Fischer titration system, Mettler DL 18 titrator or equivalent. 2. Analytical balance, capable of weighing, to the nearest 0,00001 g. 3. Hydranal Composite 5 R.D.H. as titrating solution or equivalent.		
Materials and Reagents	1. Methanol		
Preparation of Reagents	NA		
Sample Preparation	Homogenize the sample before weighing		
Method of analysis	1 Prior to titration of a sample, each working day, the titre of the employed one-component reagent (e.g. Hydranal (R)-Composite 5) is determined. 2 A suitable water standard (e.g. Hydranal R - Water Standard 10,0, ultrapure water or terpine hydrate with a moisture content well defined at 10,46%) is determined in triplicate in the employed titration medium. 3 Weigh a 1 mL syringe. Weigh approximately 30 mg of the royal jelly sample in the syringe. 4 Introduce the sample into the titration of the titrator containing about 40 mL in methanol. 5 Weigh again the syringe. 6 The weighing of royal jelly exactly introduced in the titration cell in calculated by the difference of the two weighings of the syringe. 7 After 600s of stirring, the moisture content is determined and automatically calculated by the titrator in % and mg/kg. 8 The determined titre shall be taken into account for the calculation of the water content in the sample.		
Calculation with units of expression	After 600s of stirring, the moisture content is determined and automatically calculated by the titrator in % and mg/kg.		
Inference (Qualitative Analysis)	NA		

Reference	IS/ISO 12824:2016
Approved by	Scientific Panel on Methods of Sampling and Analysis

FOOD SAFETY AND STANDARDS AUTHORITY OF INDIA Inspiring Trust, Assuring Safe & Nutritious Food Ministry of Health and Family Welfare, Government of India	**Determination of Moisture (Lyophilization Method)**		
Method No.	FSSAI 04B.030:2023	**Revision No. & Date**	0.0
Scope	Royal Jelly.		
Caution	Properly mix the sample before take it and no bubble shall be there.		
Principle	Lyophilization or freeze drying is a process in which moisture content is removed from a product after it is frozen and placed under a vacuum, allowing the ice to change directly from solid to vapor without passing through a liquid phase. After completion of lyophilization % loss of moisture is calculated.		
Apparatus/Instruments	1 Analytical balance, capable of weighing, to the nearest 0,00001 g. 2 Centrifuge tubes 3 Lyophilizer 4 Freezer		
Materials and Reagents	NA		
Preparation of Reagents	NA		
Sample Preparation	Homogenize the sample before weighing		
Method of analysis	1. Weigh accurately centrifuge tube with its cap. 2. Weigh exactly around 1 g of royal jelly in it. 3. Lyophilize at least 36 h without the cap. 4. After completion of lyophilization process, put the cap and weigh the sample immediately.		
Calculation with units of expression	The percentage of dry matter is calculated using %dry matter = 100 x $(m_1 – m_2)/m$ Where m_1 = is the mass of the tube after the lyophilization process with the cap, in grams; m_0 = is the mass of the empty tube with its cap, in grams; m = is the mass of the sample, in grams The moisture content in royal jelly is calculated using % moisture content = 100 - % dry matter		
Inference (Qualitative Analysis)	NA		
Reference	IS/ISO 12824:2016		
Approved by	Scientific Panel on Methods of Sampling and Analysis		

FOOD SAFETY AND STANDARDS AUTHORITY OF INDIA Inspiring Trust, Assuring Safe & Nutritious Food Ministry of Health and Family Welfare, Government of India	**Determination of 10-HAD (HPLC-UV External Standard: Reference method)**		
Method No.	FSSAI 04B.031:2023	**Revision No. & Date**	0.0
Scope	Royal Jelly		
Caution	Properly mix the sample before take it and no bubble shall be there.		
Principle	10-HDA is a bio-active compound found in royal jelly**.** Lyophilized royal jelly sample is extracted with phosphate buffer and 10HDA is detected by HPLC-UV at 216 nm.		
Apparatus/Instruments	1.HPLC with UV detector 2.Column: Zorbax SB-CN 150 x 3.0 mm; 3.5 µm or equivalent 3.Ultrasonic bath 4.Homogenizer 5. Analytical balance (0.00001 g)		
Materials and Reagents	1. Reference standard: 10-HDA (purity above 99%) 2. Ultrapure water 3. Methanol 4. Sodium di-hydrogen phosphate monohydrate 5. Orthophosphoric acid (H_3PO_4)		
Preparation of Reagents	1. 10-HDA, Standard stock solution = 0.13 mg/ml 2. External calibration: Prepare a standard calibration curve of 10-HAD standard with different levels 1 g/100mL, 1.5 g/100mL, 2.0 g/100mL, 2.5 g/100mL corresponding to the sample with different levels as prepared for the standard. 3. Phosphate buffer (25 mM, pH 2.5): Weigh 6.90 g sodium di-hydrogen phosphate monohydrate (M= 137.99 g/mol) into 2L measuring flask, dissolve in approximately 1800 ml H_2O, adjust pH to 2.5 with 85% H_3PO_4 and fill up to volume with water. 4. Extraction solution (25 mM phosphate buffer): Mix 550 ml 25 mM phosphate buffer, pH 2.5 with 450 ml methanol, equilibrate to room temperature. 5. Sample solvent (2 mM phosphate buffer): Mix 700 ml 25 mM phosphate buffer pH 2.5 with 300 mL methanol, equilibrate to room temperature		
Sample Preparation	1. Weigh approximately 80 mg lyophiliated royal jelly or 200 mg fresh royal jelly into a 50 ml centrifuge tube. 2. Add 40 ml extraction solution. Homogenize for approximately 10 to 20 seconds using an ultrasonic bath at 15000 rpm until royal jelly material is emulsified. Treat for 10 min in ultrasonic bath. 3. Pipette 1 ml of the homogeneous extract into a 10 mL measuring flask and fill up to volume with sample solvent. 4. Filter an aliquot of the diluted extract through membrane filter (0.45 µm) 5. Inject 20 µl into the instrument. 6. Measure the concentration against an external standard calibration curve.		
Method of analysis	**Chromatography Conditions:** Detection wavelength: 216 nm Mobile Phase A: 25 mM phosphate buffer pH 2.5 Mobile Phase B: Methanol		

	Gradient: 34% B, 0.2 – 2 min 34 - 43 % B, 2.0-9.0 min 43 - 80% B, 9.0- 10 min 34% B, 10.1- 16.0 min
Calculation with units of expression	1) Standard calibration curve Determine the equation of the straight line for a plot of peak area versus purity corrected concentration (µg/mL) of the 10-HDA standard solutions of the form: **y= ax+b** where y is the area of the 10- HDA peak a is the slope of the standard curve x is the purity corrected concentration of the standard b is the y- intercept of the standard calibration curve 2) Using the 10-HDA peak area from the sample, calculate the amount of the 10- HDA in the measuring solution from the calibration curve as follows: **$x' = (y' - b)/a$** where x' is the concentration (µg/mL) of the 10- HDA in the measuring solution of the sample; y' is the area of the 10- HDA peak in the sample. The 10- HDA content ($C_{10\text{-}DHA}$) in royal jelly (sample in g/100g) is calculated by **$(C_{10\text{-}HDA}) = x' \times 40/m$** Where x' is the calculated concentration (µg/mL) of the 10- HDA in the measuring solution of the sample; 40 is the dilution factor considering the extraction volume of 40 ml, the pipette volume used for dilution (1 mL) and the volume of the measuring flask used for dilution (10 mL) m is the actual mass of the royal jelly sample, in mg.
Inference (Qualitative Analysis)	NA
Reference	IS/ISO 12824 : 2016
Approved by	Scientific Panel on Methods of Sampling and Analysis

FOOD SAFETY AND STANDARDS AUTHORITY OF INDIA Inspiring Trust, Assuring Safe & Nutritious Food Ministry of Health and Family Welfare, Government of India	**Determination of 10-HDA (HPLC-UV Internal standard)**		
Method No.	FSSAI 04B.032:2023	**Revision No. & Date**	0.0
Scope	Royal Jelly		
Caution	Properly mix the sample before take it and no bubble shall be there.		
Principle	Sample extraction is carried out with Hydrochloric acid (HCl) and the supernatant obtained after centrifugation is analysed on HPLC-UV at 210 nm.		
Apparatus/Instruments	1. HPLC with UV detector 2. Column: 250x 4.6 mm, fill amorphous silica gel with C18 bonded stationary phase of 5 or 10 µm particle size 3. Ultrasonic bath 4. Mixer 5. Vortex mixer or equivalent 6. Analytical balance (0.00001 g)		
Materials and Reagents	1. Double distilled water 2. Methanol 3. Anhydrous alcohol 4. Trans-2-hexenoic acid (as internal standard, purity >99%) 5. 10- HDA standard (purity >99%) 6. 10- HDA standard solution, HCl (c= 0.03 M) 7. Mobile phase (Methanol + 0.03M HCl + H_2O) : 55+10+35 or (Methanol +25 mM phosphate buffer pH 2.5) : 55+45		
Preparation of Reagents	1. 10- HDA standard: Decompress and dry for 24 h in the vacuum drying oven or desiccator with concentrated sulfuric acid before it is used. 2. 10- HDA standard solution: Weigh accurately 12.5 mg of dried 10-HDA standard sample and dissolve it with anhydrous alcohol and transfer it to a 25 ml volumetric flask, dilute to the mark with anhydrous alcohol and mix evenly. 3. Internal Standard solution: Weigh accurately 650 mg of trans-2-hexenoic acid dissolve with anhydrous alcohol and transfer it to a 1000 mL volumetric flask, dilute to the mark with anhydrous alcohol and mix evenly. The concentration of the internal standard solution obtained in solution is 0.65 mg/mL. 4. HCl (0.03 M): Take 100 mL of 0.1 M HCl, add 200 ml double distilled water.		
Sample Preparation	Defreeze the sample at room temperature and stir evenly with glass rod.		
Method of analysis	1. Weigh accurately and approximately 0.5 g and put in a 50 ml volumetric flask that has been weighed already. 2. Add 1 mL of 0.03 M HCl and 2 mL water, put it on the vortex mixer and mix to dissolve the sample. 3. Add anhydrous alcohol 30 ml while shaking lightly 4. Add 10 mL internal standard solution accurately. Dilute to the mark with anhydrous alcohol and mix evenly.		

	5. Immediately put in the ultrasonic bath for 15 minutes or shake on vortex mixer for 15 minutes. 6. Centrifuge at 3000 rpm for 10 minutes and filter with 0.45 µm membrane filter if necessary. Then carry out the analysis test or store in refrigerator if analysis could not be conducted immediately. 7. Inject 10µL of sample into the instrument and measure by internal standard method. Wavelength: 210 nm Column temperature: 35 ºC Flow: 1 mL/min
Calculation with units of expression	**Determination of correction factor:** Weigh 10-HDA standard solution 0.5, 1.0, 2.0, 3.0, 4.0, 5.0 ml separately and transfer them to respective 10ml volumetric flasks. Add accurately 2ml internal standard solution, dilute to the mark with anhydrous alcohol, and mix evenly. Weigh respectively 10µl of these solutions, inject it into the instrument. Plot the mass ratio of 10- HDA per internal standard against the peak area ration of that, and draw a linear calibration curve. The 10- HDA content in royal jelly, is calculated by: $\mathbf{X_2 = F \times (A_i/A_s) \times (m_s/m_i) \times 100}$ Where X_2 is the 10- HDA content in royal jelly, %; F is the correction factor; A_i is the peak area of tested group in sample; A_s is the area of the internal standard in sample; m_s is the mass of the internal standard in grams; m_i is the mass of sample , in grams.
Inference (Qualitative Analysis)	NA
Reference	IS/ISO 12824 : 2016
Approved by	Scientific Panel on Methods of Sampling and Analysis

FOOD SAFETY AND STANDARDS AUTHORITY OF INDIA Inspiring Trust, Assuring Safe & Nutritious Food Ministry of Health and Family Welfare, Government of India	**Determination of Protein: Kjeldahl method (Automatic) (Reference method)**		
Method No.	FSSAI 04B.033:2023	**Revision No. & Date**	0.0
Scope	Royal Jelly		
Caution	Properly mix the sample before take it and no bubble shall be there.		
Principle	Nitrogen is estimated in Royal Jelly using Kjeldahl method and converted to protein In digestion step the organically bonded nitrogen is converted into ammonium ions and these ammonium ions during distillation converted into ammonia which is transferred into the receiver by means of steam distillation and the ammonia is quantitavely captured in Boric acid and the concentrarion of ammonia determined by acid base titration.		
Apparatus/Instruments	1. Analytical balance (0.0001 g) 2. Digestion block: Aluminium alloy block with adjustable temperature device for measuring and controlling block temperature (for eg. Tecator Digestion System 20, 1015 Digestor or KjelDigestor K-449, SpeedDigestor K-439 or equivalent) 3. Digestion tubes (250 mL to 300 mL) 4. Distillation units: Foss Tecator 2200, Buchi KjelMaster K-375 or equivalent to accept 250 mL to 300 mL) 5. Titration Flask (500 mL graduated Erlenmeyer flask) 6. Fume exhaust manifold (with PTFE rings seals, connected to a water aspirator in a hooded sink) 7. Nitrogen free weighing boats 8. Pipetting dispenser		
Materials and Reagents	1. Concentrated sulfuric acid, 95% to 98%, reagent grade 2. Catalyst. 3. Mixed indicator 4. Boric acid ($H_3BO_{3)}$ 5. Sodium hydroxide 6. Hydrochloric acid standard solution(0.1 mol/L)		

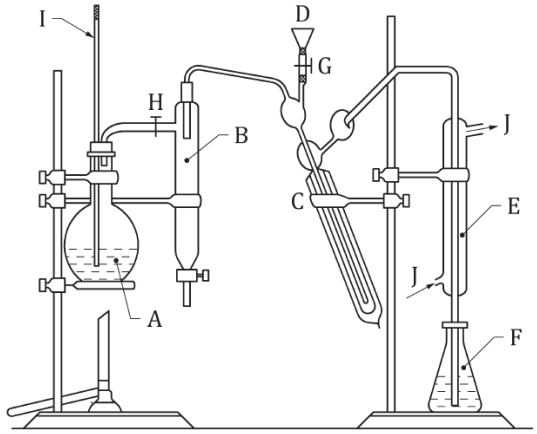
Preparation of Reagents	1. **Concentrated sulfuric acid (**95% to 98%) 2. **Catalyst**: Weigh 7.0 g potassium sulfate and 0.4 g copper sulfate. 3. **Mixed indicator:** Dissolve 100 mg methyl red in 100 ml methanol and 100 mg bromocresol green in 100 ml methanol. When potentiometric titration is used, no indicator is required. 4. **Boric acid solution**: 4% (w/v). Dissolve 400 g boric acid in 5 to 6 L hot deionized water. Mix and add more hot de-ionized water to a volume of about 9 L. Cool to room temperature, add 100 ml bromocresol green solution and 70 ml methyl red solution, and dilute to a final volume of 10 L. Adjust the pH of the boric acid solution to 4.6 to 4.8 using 0.1 mol/NaOH or 0.1 mol/L HCL or 25 ml Sheer mixed indicator and dilute to a final volume. 5. **Sodium hydroxide solution**. 32% (w/v). Weigh 32 g sodium hydroxide, dilute to100 mL with distilled water. 6. **Hydrochloric acid** standard solution,0.1 mol/ L
Sample Preparation	Homogenize the sample before weighing
Method of analysis	**Digestion: -** 1. Weigh approximately 1 g of royal jelly sample into a tarred, N free weighing boat and transfer carefully whole material into a kjeldahl tube. 2. Add the catalyst, (7.0 g potassium sulfate and 0.4 g copper sulfate) and add 12 mL of concentrated sulfuric acid, using pipetting dispenser. Hold the mixture overnight. 3. Place fume manifold tightly on tubes, and turn water aspirator on completely. 4. Place rack of tubes in preheated block (at 420 °C). 5. After 10 min, turn on water aspirator or scrubber. A condensation zone should be maintained within the tubes. After bulk of sulfur oxides fumes are produced during initial stages of digestion, reduce vaccum source to prevent loss of sulphuric acid. 6. Digest additional 50 min. Total digestion time is approximately 60 min. 7. Let tubes cool. Add deionized water to each tube to a total volume of approximately 80 ml **Distillation: -** 1. Place 32% NaOH in alkali tank of distillation unit. 2. Adjust volume dispensed to 50 mL. 3. Attach digestion tube containing diluted digest to distillation unit, or use automatic dilution feature. 4. 60 ml H_3BO_3 solution are added to the receiving vessel with indicator on receiving platform, and immerse tube from condenser below surface of H_3BO_3 solution. 5. Steam distil until ≥150 mL distillate is collected. Remove receiving flask. **Titration: -** 1. Titrate H_3BO_3 receiving solution with standard 0.1 mol/L HCl to violet or grey end point. 2. Record mL of HCl consumed to end point.
Calculation with units of expression	The protein content in royal jelly is calculated by $(V_s - V_b) \times M \times 14.01$

	$N = \frac{(Vs - Vb) \times M \times 14.01 \times 100}{m \times 10} \times 6.25$ where N = is the protein content in royal jelly, given by mass fraction, %; Vs = is the volume of standardized acid consumed when the sample is titrated, in mL; Vb = is the volume of standardized acid consumed when blank titration is made, in mL; M = is the concentration of hydrochloric acid standard solution, in mol/l; 14.01 = is the atomic weight of N; M = is the mass of sample, in grams; 10 = is the factor to convert mg/g to percent; 6.25 = is the factor to convert N to proteins.
Inference (Qualitative Analysis)	NA
Reference	IS/ISO 12824:2016
Approved by	Scientific Panel on Methods of Sampling and Analysis

FOOD SAFETY AND STANDARDS AUTHORITY OF INDIA Inspiring Trust, Assuring Safe & Nutritious Food Ministry of Health and Family Welfare, Government of India	**Determination Protein : Kjeldahl method (Alternative Method)**		
Method No.	FSSAI 04B.034:2023	**Revision No. & Date**	0.0
Scope	This method is applicable for Royal Jelly		
Caution	Properly mix the sample before take it and no bubble shall be there.		
Principle	Nitrogen is estimated in Royal Jelly using Kjeldahl method and converted to proteins. In digestion step the organically bonded nitrogen is converted into ammonium ions and these ammonium ions during distillation converted into ammonia which is transferred into the receiver by means of steam distillation and the ammonia is quantitavely captured in Boric acid and the concentrarion of ammonia determined by acid base titration.		
Apparatus/Instruments	1. **Kjeldahl nitrogen determination method digestion equipment**, 50 mL Kjeldahl flask (if far infrared digesting furnace is used, a 50 mL digesting tube and retort funnel shall be collocated). 2. **Acid burette**, 10 mL capacity 3. **Analytical balance**, Readability 0.00001 g. 4. **Semimicro method distillation unit**		
Materials and Reagents	1. Concentrated sulfuric acid, 95% to 98%, reagent grade 2. Mixed Catalyst. 3. Mixed indicator 4. Boric acid solution 5. Sodium hydroxide 6. Sulfuric acid. 7. Hydrochloric acid standard solution (0.1 mol/L)		
Preparation of Reagents	1. **Concentrated sulfuric acid**,w = 95%~98%. 2. **Mixed catalyst of copper sulfate and potassium sulfate**. Weigh 1 g copper sulfate and 10 g potassium sulfate, put it in the mortar, mix evenly, and grind finely to use. 3. **Mixed indicator**: Weigh two volumes of methyl red ethanol solution (ρ = 1 g/L) and three volumes of bromocresol green ethanol solution (ρ = 2 g/L), and mix evenly, or use mixed indicator. 4. **Boric acid absorption solution (ρ = 20 g/L**). Weigh 2.0 g boric acid, put it in the 100 ml measuring cylinder, add 20 ml ethanol, dilute to the mark with distilled water, shake until the boric acid is dissolved, and put it aside for later use. 5. **Sodium hydroxide solution (ρ = 400 g/L) :** Weigh 32 g sodium hydroxide, and dilute to 100 ml with distilled water. 6. **Dilute sulfuric acid :** Using a pipette, take 5.7 mL concentrated sulfuric acid, and dilute to 100 mL with distilled water. 7. **Hydrochloric acid standard solution (0.1 mol/L) :** Dilute to 10 times before using.		
Sample Preparation	Homogenize the sample before weighing		

Method of analysis	**Cleaning of distillation unit**

1. **Figure: Semimicro method distillation unit**



Key

A	1 000 ml round bottom flask	F	100 ml conical flask
B	safety bottle	G, H	nip for rubber tube
C	distiller connected with the ball for nitrogen	I	safety tube
D	funnel	J	water
E	condenser tube		

Link distillation unit, add proper amount of distilled water and a few drops of methyl red indicator in bottle A,

1. Add dilute sulfuric acid to make it acidic, add a few granules of glass beads and zeolites.
2. Add 50 mL distilled water from funnel D, close nip G, open condensate water, and boil the distilled water in bottle A.
3. When the vapor comes from the top of the condenser tube, remove the fire, close nip H, and make the distilled water in bottle C flow reversely to Bottle B.
4. Open nip G, discharge the distilled water in bottle B and close nip B and G.
5. Immerge the top of the condenser tube in approximate 50 ml distilled water, make the distilled water flow reversely to bottle C from the top of the condenser tube and then flow to bottle B, and discharge the distilled water with the above method.
6. Clean the apparatus twice or three times like this.

2) Digestion

1. Weigh approximately 1 g of royal jelly sample, put it on a filter paper or a paraffin paper that is weighed, pack it well after being weighed accurately, and put it in Kjeldahl flask or a digesting tube.
2. Add 2 g of mixed catalyst of copper sulfate and potassium sulfate, add 10 mL concentrated sulfuric acid slowly along the bottle wall, mix sufficiently.
3. Put a small funnel at the bottle mouth, make the flask lean at a 45° angle, heat slowly at comparative low temperature at first, keep the temperature of the solution below the boiling point, and increase the electric power gradually until the boiling is stopped.
4. When the digestion solution is boiling, maintain this state and watch out that the solution shall not overflow; heat another 30 min after the solution becomes clear green.

	5. Transfer to a 100 ml volumetric flask after it is cooled, dilute to the mark with distilled water and shake evenly for later use. **3) Distillation** 1. Weigh 10 mL boric acid of 20 g/L 2. Put it in a 100 mL conical flask, add five drops of mixed indicator, immerge the top of the condenser tube in the solution, 3. Take 5 mL of the above digestion solution accurately, move to reaction tube through funnel D, then add 10 mL sodium hydroxide of 400 g/L 4. Clean the funnel D repeated with a little distilled water, close nip G and add a few milliliters of distilled water in funnel D for the purpose of closing tube. 5. Heat bottle A (dilute sulfuric acid shall be added a drop by drop into the distilled water in the bottle so as to keep its acidity) and distil the vapor. 6. When the boric solution starts to become cyan from wine red, keep distilling for 10 min, lift the top of the condenser tube from the solution, make the vapor continue to wash for 1 min, drip-washing the top with a little distilled water and stop distillation. **4) Titration: -** 1. The absorption solution shall be titrated with 0.01 mol/hydrochloric acid standard solution. 2. When the color changes from cyan to grey purple, the end point has been reached.
Calculation with units of expression	The protein content in royal jelly is calculated by $$X_3 = \frac{(V_1 - V_0) \times c_1 \times 0.014}{m_4 \times 5/100} \times 6.25 \times 100$$ where X_3 = is the protein content in royal jelly, given by mass fraction, %; V_1 = is the volume of 0.01 mol/L hydrochloric acid standard solution consumed when the sample is titrated, in millilitres; V_0 = is the volume of 0.01 mol/L hydrochloric acid standard solution consumed when blank titration is made, in millilitre; c_1 = is the concentration of hydrochloric acid solution, in mol/L; 0.014 = is the millimol mass of nitrogen, in grams; m_4 = is the mass of sample, in grams; 6.25 = is the coefficient of protein conversed from nitrogen.
Inference (Qualitative Analysis)	NA
Reference	IS/ISO 12824:2016
Approved by	Scientific Panel on Methods of Sampling and Analysis

	Determination of Sugar: Titration Method		
Method No.	FSSAI 04B.035:2023	**Revision No. & Date**	0.0
Scope	This method is applicable for Royal Jelly		
Caution	Properly mix the sample before take it and no bubble shall be there.		
Principle	This method is involving the reduction of solution A and Solution B by titration at boiling point against a solution of reducing sugar in honey by using methylene red as internal indicator last faded blue color of the sample noted as final reading to calculate the Sugars.		
Apparatus/Instruments	Electric-headed thermostatic water bath: (±1°C). Analytical balance: (0.0001g)		
Materials and Reagents	1. Glucose standard Alkaline cupric tartrate solution A, 2. Alkaline cupric tartrate solution B, 3. Zinc acetate solution, 4. Potassium ferrocyanide, 5. Hydrochloric acid 6. Hydrochloric acid 7. Sodium hydroxide Methyl red indicator.		
Preparation of Reagents	**1. Glucose standard solution:** Weigh accurately 1000 g pure glucose (specific rotation is +52.5 ~ +53°) with constant weight after it is dried at the temperature from 98°C to 100°C, dissolve with distilled water and add 5 mL hydrochloric acid (c=6 mol/L) and dilute to 1000 mL with distilled water. 2. **Alkaline cupric tartrate TS solution A:** Dissolve 15 g copper sulfate ($CuSO_4.5H_2O$) and 0.05g methylene blue, in 1000 mL water, and store in a tightly stoppered bottle. 3. **Alkaline cupric tartrate TS solution B**: Weigh 50 g potassium sodium tartrate and 75 g sodium hydroxide, dissolve with distilled water, add 4 g potassium ferrocyanide, dilute to 1000 mL with distilled water when it is dissolved completely and store in a tightly stoppered polyethylene plastic bottle. Calibration of alkaline cupric tartrate TS solution: weigh accurately 5 mL respectively from alkaline cupric tartrate TS solution A and B, put them in 150 mL conical bottles, add 10 ml distilled water, add approximately 9 ml glucose standard solution from burette, heat to the boiling point within 2 min and keep adding glucose standard solution at the speed of one drop per 2 s when it is boiling. The end point is reached when the blue colour of the solution has just faded. Record the total volume of the glucose standard solution consumed, operate three times in parallel at the same time, take the mean value and calculate the mass (mg) of the glucose equivalent to 10 ml (5 ml per respectively from solution A and B) of alkaline cupric tartrate TS solution.		

	4. Zinc acetate solution, ρ = 219 g/L. Weigh 21.9 g zinc acetate, add 3 mL acetic acid, dissolve with distilled water and dilute to 100 mL. 5. Potassium ferrocyanide, ρ = 106 g/L. 6. Concentrated hydrochloric acid, w = 36 % ~ 38 %. 7. Hydrochloric acid, c = 6 mol/L. Weigh 50 ml hydrochloric acid, add distilled water and dilute to 100 mL. 8. Sodium hydroxide solution, ρ = 200 g/ L Methyl red indicator, ρ = 1 g/L, ethanol solution.
Sample Preparation	1. Weight approximately 4 g of royal jelly sample; put it in a 100 mL volumetric flask. 2. Add 50 mL distilled water; shake till dissolution of the sample. 3. Then add 5 mL zinc acetate solution and potassium sodium tartrate respectively and slowly, dilute to the mark with distilled water, and mix evenly. 4. Allow standing for 30 min and filtrating with dried filter paper, discard a few milliliters of initial filtrate. The filtrate is for later use.
Method of analysis	1. Take accurately 50 mL of the above filtrate, put it in a 100 mL volumetric flask, add 10 mL hydrochloric acid (c = 6 mol/L), mix evenly, put it in an electric-heated thermostatic water bath, hydrolyze for 10 min at the temperature from 68 °C to 70 °C, leave it to room temperature by cooling with flowing water, add two drops of methyl red indicator and mix evenly, neutralize with sodium hydroxide (p = 200 g/L) until the solution becomes yellow and dilute to the mark with distilled water and mix evenly, which serves as sample solution and is prepared for later use. 2. Take accurately 5 mL of alkaline cupric tartrate TS solution A and B respectively. 3. Put them in 150 mL conical bottles, heat to the boiling point within 2 min, at a speed that is fast at first and slow later. 4. Add sample solution drop by drop from the burette and keep the solution in boiling state. 5. When the solution colour starts to lose, titrate at the speed of one drop per 2 seconds. 6. The end point is reached when the colour blue has just faded. 7. Record the volume of the sample solution consumed.
Calculation with units of expression	The total sugar content in royal jelly is calculated by: $X_4 = \frac{T}{m_5 \times V_2/100 \times 1/2 \times 1000} \times 100$ Where, X_4 is the total sugar content (counted by glucose), given by mass fraction, %; T is the titre value of alkaline cupric tartrate TS, the mass of which 10 ml alkaline cupric tartrate TS (5 ml respectively from solution A and B) equals to glucose, in milligrams; m_5 is the mass of the sample, in grams; V_2 is the volume of sample solution consumed in titration, in milliliters.
Inference (Qualitative Analysis)	NA
Reference	IS/ISO 12824 : 2016
Approved by	Scientific Panel on Methods of Sampling and Analysis

	Determination of Sugars : Fructose, Glucose, Sucrose, Erlose, Maltose and Maltotriose (by HPLC : Reference Method)		
Method No.	FSSAI 04B.036:2023	**Revision No. & Date**	0.0
Scope	This method is applicable for the determination of Fructose, Glucose, Sucrose, Erlose, Maltose, Maltotriose in Royal Jelly		
Caution	Properly mix the sample before take it and no bubble shall be there.		
Principle	Sugar is extracted from the sample by mixing it with methanol & water and the supernatant collected after centrifugation is analysed on RID detector for various Sugars estimation.		
Apparatus/Instruments	1. HPLC with refractive index detector (RID) 2. Column: Amino modified phase 3. Ultrasonic bath 4. Centrifuge 5. Analytical balance (0.00001 g)		
Materials and Reagents	1. Acetonitrile (HPLC grade) 2. Methanol (HPLC grade) 3. Ultra pure water 4. Sugar standards (≥98.0 % purity)		
Preparation of Reagents	Standard (M): Weigh exactly the sugar standard in order to obtain in anhydrous sugar concentration of 1g/100mL. Transfer in a 100mL flask. Add around 25mL of water and stir. Make up the volume with methanol. F1: Dilute 10mL of solution M in a 20mL volumetric flask with a mixture MeOH/H_2O:75/25 F2: Dilute 5mL of solution M in a 20mL volumetric flask with a mixture MeOH/H_2O:75/25		
Sample Preparation	1. Weigh accurately and approximately 2 g of royal jelly in a beaker. 2. Add some milliliters of a solution MeOH/H_2O: 75/25 under magnetic stirring 3. Transfer in a 20 mL volumetric flask and complete with the same solution MeOH/H_2O 4. Centrifuge for 10 min at a speed of 4000 rpm. 5. Filter the supernatant before chromatographic injection.		
Method of analysis	1. Mobile Phase : Acetonitrile : water (75:25) 2. Flow: 1 mL/min 3. Column Temp. : 30ºC		
Calculation with units of expression	The concentration of the sugar *i* in sample is calculated using formula: $\mathbf{C_i = k_i + A_i}$ **Where** $\mathbf{C_i}$ is the concentration of the sugar *I* in sample; in mg/mL; $\boldsymbol{k_i}$ is the response factor of sugar *i*, which is calculated from the slope of the calibration curve constructed by the area against concentration of the standard solutions (M, F1, F2); $\mathbf{A_i}$ is the area of sugar *i* in sample.		

	Total sugar in royal jelly is calculated using formula: **%Sugar *i* = C_i × 20/m × 100** Where %Sugar *i* is the percentage of the sugar *i* in royal jelly; C_i is the concentration of the sugar *i* in sample; in mg/mL; M is the mass of the sample, in mg. % Total sugar = % Sugar (Fructose + glucose+ Sucrose)
Inference (Qualitative Analysis)	NA
Reference	IS/ISO 12824 : 2016
Approved by	Scientific Panel on Methods of Sampling and Analysis

FOOD SAFETY AND STANDARDS AUTHORITY OF INDIA Inspiring Trust, Assuring Safe & Nutritious Food Ministry of Health and Family Welfare, Government of India	**Determination of Sugar : Fructose, Glucose, Sucrose, Erlose, Maltose and Maltotriose (By Gas Chromatography)**		
Method No.	FSSAI 04B.037:2023	**Revision No. & Date**	0.0
Scope	Royal Jelly		
Caution	Properly mix the sample before take it and no bubble shall be there.		
Principle	Sugar is extracted from the sample by mixing it with Pyridine, hexamethyldisilazane and Trimethylchlorosilane. and analyzed on FID detector for various Sugars estimation.		
Apparatus/Instruments	1. GC with flame ionization detector 2. Chromatographic column HP5-MS column (30 m x 0.25 mm x 0.25µm) 3. Analytical balance (0.00001 g)		
Materials and Reagents	1. Hexamethyldisilazane (≥ 99% purity) 2. Trimethylchlorosilane (≥ 99% purity) 3. Pyridine (≥ 99.8 % purity) (anhydrous pyridine is obtained by distillation over calcium hydride) 4. Sorbitol (internal standard) (≥ 99% purity)		
Preparation of Reagents	Anhydrous pyridine is obtained by distillation over calcium hydride.		
Sample Preparation	1. Weigh accurately about 40 mg of lyophilized royal jelly and 1 mg of sorbitol. 2. Introduce them in a glass reactor and close tightly. 3. Then add 1 mL of anhydrous pyridine. Stir the mixture for 5 minutes with the reactor sealed.\ 4. Then add 200 µl of hexamethyldisilazane and stir the mixture for 5 minutes. 5. Add 100 µL trimethylchlorosilane and stir for 30 minutes. 6. Leave the mixture for 20 h at room temperature with the reactor sealed.		
Method of analysis	1. Helium as carrier gas (5.0 grade) constant pressure of 22 psi 2. Injection volume: 2 µL 3. Injection and detector temperatures set at 280 ºC 4. Program of oven temperature: Maintain initial temperature (150 ºC) for 5 minutes, then increase to 325 ºC at a rate of 3 ºC/min 5. Maintain the final temperature for 10 min. 6. Use reference standards or retention indics to identify the different sugars. Determine the retention indics of each sugar by injecting the standard with the same analytical and chromatographic conditions.		
Calculation with units of expression	1. **Sugar Quantification-Determination of correction factor**: Sugar is quantified by internal standard (Sorbitol). A response factor or mass correction factor is calculated for each sugar by following fomula: $k_i = A_{SI}/A_i \times M_i/M_{SI}$ Where, k_i is the response factor of the sugar *i* A_{SI} is the area of the internal standard A_i is the area of the standard of sugar *i* M_{SI} is the mass of the internal standard M_i is the mass of the standard of sugar *i* 2. Calculations: The mass of the sugar *I* in the royal jelly sample is calculated using formula.		

	$M_i = k_i \times A_i / A_{SI} \times m_{SI}$ where m_i is the mass of the sugar *I* in the royal jelly sample in mg; k_i is the response factor of sugar *I;* A_{SI} is the area of the internal standard; A_i is the area of sugar *I* in the royal jelly sample' M_{SI} is the mass of the internal standard, in mg. The percentage of the sugar *I* in the royal jelly is calculated using the formula: $\%\ \text{sugar}_I = \%\ MS \times m_i / m_{sample}$ Where m_i is the mass of the sugar *I* in the royal jelly sample in mg; m_{sample} is the mass of the royal jelly sample, in mg; %MS is the dry matter percentage.
Inference (Qualitative Analysis)	NA
Reference	IS/ISO 12824 : 2016
Approved by	Scientific Panel on Methods of Sampling and Analysis

FOOD SAFETY AND STANDARDS AUTHORITY OF INDIA Inspiring Trust, Assuring Safe & Nutritious Food Ministry of Health and Family Welfare, Government of India	**Determination of Total Acidity**		
Method No.	FSSAI 04B.038:2023	**Revision No. & Date**	0.0
Scope	Royal Jelly		
Caution	Properly mix the sample before take it and no bubble shall be there.		
Principle	The total acidity is the sum of the free acidity and the lactone acidity. The total acidity is obtained by adding an excess of sodium hydroxide to the honey solution and the end point is achieved when the pH-meter indicates at pH 8.3.		
Apparatus/Instruments	1. pH-meter pH value,to the nearest 0.1 2. Burette,10 mL 3. Analytical balance,capable of weighing to the nearest 0.0001 g.		
Materials and Reagents	Sodium hydroxide		
Preparation of Reagents	Sodium hydroxide,c = 0.1 mol/L		
Sample Preparation	Homogenize the sample before weigh		
Method of analysis	1. Weigh 1.0 g royal jelly sample. 2. Put it in a 100 mL beaker, and add 75 mL boiled and cooled distilled water. 3. Titrate with sodium hydroxide standard solution (c = 0.1 mol/L). 4. The end point is achieved when the pH-meter indicates at pH 8.3.		
Calculation with units of expression	The millilitre quantity of sodium hydroxide standard solution consumed in titration is multiplied by the concentration value (mol/L) and divided by the mass of sample, and then multiplied by 100. The acidity of sample is determined. Acidity [(1 mol/NaOH) ml/100 g] = (V x c x 100)/m Where V = is the volume of 0.1 mol/L NaOH standard solution consumed in titration, in millilitres; C = is the concentration of NaOH standard solution, in mol/L; M = is the mass of sample, in grams.		
Inference (Qualitative Analysis)	NA		
Reference	IS/ISO 12824 : 2016		
Approved by	Scientific Panel on Methods of Sampling and Analysis		

FOOD SAFETY AND STANDARDS AUTHORITY OF INDIA Inspiring Trust, Assuring Safe & Nutritious Food Ministry of Health and Family Welfare, Government of India	**Determination of Total Lipid**		
Method No.	FSSAI 04B.039:2023	**Revision No. & Date**	0.0
Scope	Royal Jelly		
Caution	Properly mix the sample before take it and no bubble shall be there.		
Principle	Fat extractor uses the Diethyl ether reflux and siphon principle to continuously extract the solid matter, and calculate the fat by difference between the initial and final weight of beaker.		
Apparatus/Instruments	1. Soxhlet extraction apparatus, with soxhlet extraction tube (internal diameter ca. 40 mm), extraction bottle and condenser tube. 2. Thimble Filter, of internal diameter 25 mm to 30 mm, length 100 mm to 120 mm. 3. Thermostatic bath 4. Drying Oven 5. Vaccum drying oven.		
Materials and Reagents	2) **Diethyl ether** 3) **Celite**		
Preparation of Reagents	1) **Diethyl ether**, of purity above 99.5%. Or use tert-buthylmethyl ether (TBME) as alternative extraction solvent.		
Sample Preparation	Homogenize the sample before weighing.		
Method of analysis	1. Weigh accurately approximately 2.5 g of royal jelly sample in a beaker and add 3 g to 5 g of Celite. 2. Mix the sample and Celite well with a glass rod until the mixture is equalized. 3. Transfer the mixture from the beaker to thimble filter and wipe carefully the beaker and the glass rod with defatted cotton impregnated with diethyl ether 4. Put the defatted cotton into upper half of thimble filter. 5. Dry in air the thimble filter until the smell of diethyl ether has gone. 6. Dry the thimble filter for 2 h at 70 °C under the pressure in vaccum drying oven. 7. Add 100 mL to 150 mL diethyl ether into an extraction bottle which is dried until a constant weight, put the thimble filter into extraction tube, and connect the extraction tube to a condenser tube and the extraction bottle. 8. Extract lipid on a thermostatic bath at approximately 50 °C for 8 h. 9. After extraction, take the thimble filter out of the extraction tube, evaporate almost all the diethyl ether in the extraction bottle and completely evaporate it by evaporator or nitrogen gas. 10. Wipe the outside of the extraction bottle. 11. Dry it in a drying oven at 105 °C for 1 h and weigh it after cooling in a desiccator for 1 h.		
Calculation with units of expression	The total lipid in royal jelly is calculated by $X_5 = \frac{m_7 - m_6}{m_8} \times 100$		

	where X_5 = is the total lipid content, given by mass fraction, %; m_6 = is the mass of the extraction bottle which is dried until the constant weigh, in grams; m_7 = is the mass of the extraction bottle after extraction and drying, in grams; m_8 = is the mass of the sample, in grams.
Inference (Qualitative Analysis)	NA
Reference	IS/ISO 12824 : 2016
Approved by	Scientific Panel on Methods of Sampling and Analysis

FOOD SAFETY AND STANDARDS AUTHORITY OF INDIA Inspiring Trust, Assuring Safe & Nutritious Food Ministry of Health and Family Welfare, Government of India	**Determination of $\delta^{13}C/\delta^{12}C$ Isotopic Ratio**		
Method No.	FSSAI 04B.040:2023	**Revision No. & Date**	0.0
Scope	Royal Jelly		
Caution	Properly mix the sample before take it and no bubble shall be there. Samples must be folded properly in the tin capsule to avoid sample leakage and air trap.		
Principle	Sample injected into the Elemental analyzer (EA) is combusted and oxidized and the CO_2 produced from combustion of the bulk royal jelly is quantified in the form of carbon isotopic value of $^{13}C/^{12}C$ ratio by Ion ratio mass spectrometer (IRMS)		
Apparatus/Instruments	1. Elemental Analyzer 2. Ion ratio mass spectrometer (IRMS) 3. Analytical balance (0.00001g) 4. Tin capsules 5. Blunt ended forceps		
Materials and Reagents	1. Chromium Oxide 2. Cobaltous/Cobaltic Oxide		
Preparation of Reagents	NA		
Sample Preparation	100 to 1000 µg of royal jelly are loaded into a tin (or silver) capsule.		
Method of analysis	1. Samples are dropped from a carousel-type auto sampler into a reactor filled with chromium oxide and cobaltous /cobaltic oxide. 2. Automated oxygen dosing ensures complete combustion of the sample. Subsequent to combustion, NOx compounds are reduced to N2 in a reactor filled with reduced copper. 3. All gas species are carried in a continuous helium stream and separated on an isothermal GC column.H_2O and SOx species are removed by adsorption.		
Calculation with units of expression	The CO_2 produced from combustion of the bulk royal jelly is analysed for the $^{13}C/^{12}C$ ratio in a dedicated isotopic ratio mass spectrometer.		
Inference (Qualitative Analysis)	NA		
Reference	IS/ISO 12824 : 2016		

	Determination of Furosine		
Method No.	FSSAI 04B.041:2023	**Revision No. & Date**	0.0
Scope	Royal Jelly		
Caution	Properly mix the sample before take it and no bubble shall be there.		
Principle	Acid hydrolyzed sample is loaded on conditioned SPE cartridge and finally eluted with hydrochloric acid and injected into the instrument for final quantification.		
Apparatus/Instruments	1. HPLC with UV detector (or DAD) 2. Analytical balance (0.00001 g)		
Materials and Reagents	1. Sodium Acetate 2. Acetic Acid 3. Hydrochloric acid (HCl) 4. Syringe-tip filter: 0.45 µm PTFE seal or equivalent. 3. SPE cartridge: C18, 500 mg (SPE-PAK cartridge) or equivalent. 4. Column: Reverse phase C-8, 25 cm x 4.6 mm, 5 µm or equivalent 5. Vial: Amber glass vial		
Preparation of Reagents	1. 0.06 M/L Sodium Acetate, pH 4.3 with acetic acid: 4.92g Sodium acetate in 1000 mL of water. 2. 3 M/L HCl: Take 250 mL of 12 M HCl and make up with 1000 ml distilled water 3. 8 M/L HCl: Take 666 mL of 12 M HCl and make up with 1000 ml distilled water		
Sample Preparation	1. An aliquot of sample (0.35 g) corresponding to about 30mg to 70mg of protein, is hydrolyzed with 8ml of 8 M/L HCl at 110 °C for 23 h. 2. After hydrolysis, collect 0.5 mL of hydrolysate. 3. SPE C18 cartridge conditioning: Conditioning the SPE cartridge with 5 ml methanol followed with 10 mL ultrapure water. 4. Load 0.5 mL hydrolysate sample on the SPE C18 cartridge. 5. Discard the eluate and Dry the cartridge in air. 6. Elute 1 mL x 4 of HCl 3M/L 7. Collect all the eluate in a 5 mL volumetric amber glass and make up with 5 mL 3 M/L HCl solution. 8. Filter with syringe-tip filter (0.45µm) in amber glass vial. 9. Inject on 50µl in a HPLC for analysis. **Protein Determination** Follow the method FSSAI 04B.033:2023/FSSAI 04B.034:2023		
Method of analysis	1. Mobile phase:0.06 M/L sodium acetate, pH 4.3 acetic acid 2. Flow: 2 mL/min 3. Column Temperature: 30 °C 4. Detector: UV-280 nm 5. Injection volume:20 µL to 50 µL		
Calculation with units of expression	Quantification the Furosine by external calibration standard and express the value as: Furosine = mg Furosine/100g protein		

Inference (Qualitative Analysis)	NA
Reference	IS/ISO 12824 : 2016
Approved by	Scientific Panel on Methods of Sampling and Analysis