BS ISO 12824:2016

BSI Standards Publication

Royal jelly — Specifications

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National foreword

This British Standard is the UK implementation of ISO 12824:2016.

The UK participation in its preparation was entrusted to Technical Committee AW/-/2, Food Technical Committee Chairmen.

A list of organizations represented on this committee can be obtained on request to its secretary.

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Published by BSI Standards Limited 2016

ISBN 978 0 580 88352 1

ICS 67.180.10

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This British Standard was published under the authority of the Standards Policy and Strategy Committee on 30 September 2016.

Amendments/corrigenda issued since publication

Date	Text affected

BS ISO 12824:2016

INTERNATIONAL STANDARD

ISO 12824

First edition
2016-09-15

Royal jelly — Specifications

Gelée royale — Spécifications

Reference number
ISO 12824:2016(E)

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Foreword

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The procedures used to develop this document and those intended for its further maintenance are described in the ISO/IEC Directives, Part 1. In particular the different approval criteria needed for the different types of ISO documents should be noted. This document was drafted in accordance with the editorial rules of the ISO/IEC Directives, Part 2 (see www.iso.org/directives).

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For an explanation on the meaning of ISO specific terms and expressions related to conformity assessment, as well as information about ISO's adherence to the WTO principles in the Technical Barriers to Trade (TBT) see the following URL: Foreword - Supplementary information.

The committee responsible for this document is ISO/TC 34, *Food products*.

INTERNATIONAL STANDARD ISO 12824:2016(E)

Royal jelly — Specifications

1 Scope

This International Standard specifies the production and sanitary requirements for royal jelly and establishes a series of organoleptic and chemical test methods to control royal jelly quality. It also specifies the requirements of transport, storage, packaging and marking for royal jelly. This International Standard applies to the royal jelly production (collecting, preliminary processing and packaging) and trade links. This International Standard is not applicable to royal jelly products in which other foods are mixed.

2 Normative references

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

ISO 4833-1, *Microbiology of the food chain — Horizontal method for the enumeration of microorganisms — Part 1: Colony count at 30 °C by the pour plate technique*

ISO 21528-2, *Microbiology of food and animal feeding stuffs — Horizontal methods for the detection and enumeration of Enterobacteriaceae – Part 2: Colony-count method*

ISO 6579, *Microbiology of food and animal feeding stuffs — Horizontal method for the detection of Salmonella spp.*

3 Terms and definitions

For the purposes of this document, the following terms and definitions apply.

3.1
royal jelly
mixture of secretions from hypopharyngeal and mandibular glands of worker bees, free from any additive

Note 1 to entry: It is the food of larval and adult queens. It is a raw and natural food, unprocessed except for filtration which does not undergo addition of substances. The colour, the taste and the chemical composition of royal jelly are determined by absorption and transformation by the bees fed with the following two types of foods during the royal jelly production time:

— type 1: only bee's natural foods (pollen, nectar and honey);

— type 2: bee's natural food and other nutrients (proteins, carbohydrates, etc.).

3.2
10-HDA
10-hydroxy-*2*-decenoic acid
characteristic material of royal jelly

4 Requirements

4.1 Description

Royal jelly is milky white, pale yellow, with luster. It is pasty or jelly-like at room temperature with fluidity and shall be free from bubbles and foreign substances. Minor crystallization phenomena can occur naturally in royal jelly during storage.

4.2 Odour and taste

It is pungent, unfermented and shall not be rancescent. It is acerb, spicy and it brings acrid taste to palate and throat.

4.3 Chemical requirements

Royal jelly shall comply with the requirements given in Table 1.

Table 1 — Chemical requirements of royal jelly

Characteristic		Requirement		Analysis method
		Type 1	Type 2	
Moisture content (%)	min.	62,0		Annex A
	max.	68,5		
10-HDA(%)	min.	1,4		Annex B
Protein %	min.	11		Annex C
	max.	18		
Total sugar %	min.	7		Annex D
	max.	18		
Fructose %		2–9		Annex D
Glucose %		2–9		Annex D
Sucrose %		<3,0	Na[a]	Annex D
Erlose %		<0,5	Na[a]	Annex D
Maltose %		<1,5	Na[a]	Annex D
Maltotriose %		<0,5	Na[a]	Annex D
Total acidity [(1 mol/l NaOH) ml/100g]	min.	30,0		Annex E
	max.	53,0		
Total lipid (%)		2		Annex F
		8		
C13/C12 Isotopic ratio (δ ‰)		–29 to –20	–29 to –14	Annex G

[a] Na = Not applicable.

Furosine is an additional, optional quality parameter which shows freshness of royal jelly (see informative method in Annex H).

NOTE A value is to be specified in the next revision of this International Standard.

Pollen screening may be used to determine geographical origin of royal jelly (see informative method in Annex I).

4.4 Hygienic requirements

Royal jelly shall comply with the requirements given in Table 2.

Table 2 — Hygienic requirements of royal jelly

Characteristic		Requirement	Analysis method
Colony count (cfu/g)	max.	500	ISO 4833-1
Pathogenic bacteria:			
Enterobacteriaceae (cfu/g)		absent in 10 g	ISO 21528-2
Salmonella (cfu/g)		absent in 25 g	ISO 6579

5 Test methods

5.1 General

Use only reagents of recognized analytical grade, unless otherwise specified, and distilled water or water of equivalent purity.

5.2 Sample collection

Sample collector shall use stainless steel bar, tube or spoon. Put the sample into the sterile sample bottle, stir sufficiently in order to mix it evenly, and put it aside as the sample to be tested. Each sample shall not be less than 20 g.

The sample shall be tested immediately or it shall be stored in a refrigerator below 5 °C.

5.3 Test methods of chemical requirements

Samples shall be tested according to the test methods specified in Annex A, Annex B, Annex C, Annex D, Annex E, Annex F and Annex G or any other test methods with performances recognized as at least equivalent according to recognized standards.

6 Packaging, marking, storage and transportation

6.1 Packaging

Packaging in contact with royal jelly shall be of food grade.

6.2 Marking

At least the following information shall be marked on each package or on a label:

a) the name of the product, and trade name or brand name, if any;

b) the name and address of the producer or packer;

c) the net weight;

d) the harvesting country/countries;

e) the harvesting year;

f) the date of minimum durability;

g) the storage mode and instructions;

h) the freezing month if any;

i) the type, according to this International Standard;

k) the batch number.

6.3 Storage and transportation

The temperature for storage shall be between +2 °C and +5 °C or, preferably, less than –18 °C for long-term storage.

Royal jelly produced in different areas and times should be stored separately in giving them different batch numbers (in bottle or in box).

It shall be transported at low temperature and shall not be stored and transported with toxic, corrosive material or material with peculiar smell or that might cause contamination.

Annex A
(normative)

Determination of moisture content

A.1 Vacuum drying oven method (Reference method)

A.1.1 Apparatus

A.1.1.1 Vacuum drying oven.

A.1.1.2 Weighing dish, of height 25 mm to ~30 mm, of diameter 35 mm to 50 mm.

A.1.1.3 Analytical balance, capable of weighing to the nearest 0,000 1 g.

A.1.2 Procedure

Weigh approximately 0,5 g of the royal jelly sample, put it in the weighing dish which is dried to constant weight, spread evenly, weigh accurately and put it in the vacuum drying oven, dry for 4 h at 75 °C and under the pressure between 0,000 MPa and 0,005 MPa, take out the weighing dish and put it in the drying oven or desiccator, weigh after it has been cooled for 30 min, redry for 2 h and repeat the process until the weight difference between two consecutive times is no more than 2 mg, namely, until a constant weight is achieved.

A.1.3 Calculation

The moisture content in royal jelly, X_1, expressed as a percentage by mass, is given by Formula (A.1):

$$X_1 = \frac{m_1 - m_2}{m_1 - m_3} \times 100 \quad \text{(A.1)}$$

where

X_1 is the moisture content in royal jelly, %;

m_1 is the mass of the weighing dish and the sample, in grams;

m_2 is the mass of the weighing dish and the sample that is dried until a constant weight is achieved, in grams;

m_3 is the mass of the weighing dish, in grams.

A.1.4 Precision

Relative deviation of parallel experiments shall not be more than 0,8 %.

A.2 Karl Fisher

A.2.1 Apparatus

A.2.1.1 Karl Fischer titration system, Mettler DL 18 titrator[1)] or equivalent.

A.2.1.2 Analytical balance, capable of weighing, to the nearest 0,000 01 g.

A.2.1.3 Hydranal Composite 5 R.D.H.[1)], as titrating solution or equivalent.

A.2.1.4 Methanol, UV purity or analytical purity, as solvent.

A.2.2 Procedure

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. 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.

Weigh a 1 ml syringe. Weigh approximately 30 mg of the royal jelly sample in the syringe.

Introduce the sample into the titration cell of the titrator containing about 40 ml of methanol.

Weigh again the syringe.

The weighing of royal jelly exactly introduced in the titration cell in calculated by the difference of the two weighings of the syringe.

After 600 s of stirring, the moisture content is determined and automatically calculated by the titrator in % and mg/kg.

The determined titre shall be taken into account for the calculation of the water content in the sample.

A.2.3 Precision

Each sample shall be analysed twice and the relative deviation between both measures shall not be more than 0,4 %.

A.3 Lyophilization

See Reference [1].

A.3.1 Apparatus

A.3.1.1 Analytical balance, capable of weighing to the nearest 0,000 1 g.

A.3.1.2 Centrifuge tubes.

A.3.1.3 Lyophilizer.

A.3.1.4 Freezer.

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1) Example of a suitable product available commercially. This information is given for the convenience of users of this document and does not constitute an endorsement by ISO of this product.

A.3.2 Procedure

Weigh a centrifuge tube with its cap. Weigh exactly around 1 g of royal jelly in it. Lyophilize at least 36 h, without the cap. As soon as the lyophilization process is stopped, put the cap and weigh the sample immediately.

A.3.3 Calculation

The percentage of dry matter is calculated using Formula (A.2):

$$\% \text{ dry matter} = 100 \times (m_1 - m_0)/m \quad \text{(A.2)}$$

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 Formula (A.3):

$$\% \text{ moisture content} = 100 - \% \text{ dry matter} \quad \text{(A.3)}$$

A.3.4 Precision

Relative deviation of parallel experiments shall not be more than 0,8 %.

Annex B
(normative)

Determination of 10-HDA

B.1 HPLC-UV External Standard (Reference method)

B.1.1 Reagents

Use only ultrapure water.

B.1.1.1 Methanol, UV purity or analytical purity with light transmittance above 30 % at detection wavelength.

B.1.1.2 10-HDA reference standard, of purity above 99,0 % (certificate of analysis of supplier).

B.1.1.3 10-HDA S_0 standard stock solution, c = 0,13 mg/ml.

For example, accurately weigh approximately 6,50 mg reference substance into 50 ml measuring flask, dissolve in methanol and fill up to volume with methanol.

For dilution: factor x (standard S_0) = 0,13 (mg/ml)/calculated concentration S_0 (mg/ml). The calculated concentration shall be corrected for the purity of the reference material.

B.1.1.4 25 mm phosphate buffer pH 2,5, as Eluent A, for extraction solution and sample solvent).

For example, weigh 6,90 g sodium dihydrogen phosphate monohydrate (M = 137,99 g/mol) into 2l measuring flask, dissolve in approximately 1 800 ml H_2O, adjust pH to 2,5 with 85 % H_3PO_4 and fill up to volume with H_2O.

B.1.1.5 Extraction solution, 55 % 25 mm phosphate buffer pH 2,5/45 % methanol (v:v).

For example, mix 550 ml 25 mm phosphate buffer pH 2,5 with 450 ml methanol, equilibrate to room temperature.

B.1.1.6 Sample solvent, 70 % 25 mm phosphate buffer pH 2,5/30 % methanol (v:v).

For example, mix 700 ml 25 mm phosphate buffer pH 2,5 with 300 ml methanol, equilibrate to room temperature.

B.1.2 Apparatus

B.1.2.1 HPLC with ultraviolet detector, recorder or microprocessor.

B.1.2.2 Chromatographic column, Zorbax SB-CN 150 × 3,0 mm; 3,5 µm or equivalent.

B.1.2.3 Ultrasonic bath.

B.1.2.4 Homogenizer, Ultraturrax or equivalent.

B.1.2.5 Analytical balance, capable of weighing to the nearest 0,000 01 g.

B.1.3 Procedure

B.1.3.1 Sample treatment

Exactly weigh approximately 80,00 mg lyophiliated royal jelly or 200 mg fresh royal jelly into a 50 ml centrifuge tube.

Add 40,0 ml extraction solution. Homogenize for approximately 10 s to 20 s using an ultraturrax at 15 000 rpm until all royal jelly material is emulsified. Treat for 10 min in ultrasonic bath.

Pipette 1 ml of the homogeneous extract into a 10 ml measuring flask and fill up to volume with sample solvent. Filter an aliquot of the diluted extract through membrane filter (0,45 µm).

B.1.3.2 Chromatography conditions

Detection wavelength: 216 nm

Eluent A: 25 mm phosphate buffer pH 2,5

Eluent B: Methanol

Gradient:	34 % B,	0-2,0 min
	34-43 % B,	2,0-9,0 min
	43-80 % B,	9,0-10,0 min
	34 % B	10,1 min-16,0 min

B.1.3.3 External calibration

Realize an external standard calibration curve (solutions corresponding to concentration of 10-HDA: 1,0 g/100 ml, 1,5 g/100 ml, 2,0 g/100 ml, 2,5 g/100 ml).

The calibration curve shall be linear by visual assessment with a coefficient of correlation $r > 0,99$.

Standard dilutions for calibration curve:

Standard 10-HDA 1,0 g/100 ml: c = 5 µg/ml (corresponds to 1,0 g/100 g in sample)	Pipette (x * 385) µl 10-HDA-S_0 standard stock solution into a 10 ml measuring flask and dilute to volume with sample solvent
Standard 10-HDA 1,5 g/100 ml: c = 7,5 µg/ml (corresponds to 1,5 g/100 g in sample)	Pipette (x * 578) µl 10-HDA-S_0 standard stock solution into a 10 ml measuring flask and dilute to volume with sample solvent
Standard 10-HDA 2,0 g/100 ml: c = 10 µg/ml (corresponds to 2,0 g/100 g in sample)	Pipette (x * 770) µl 10-HDA-S_0 standard stock solution into a 10 ml measuring flask and dilute to volume with sample solvent
Standard 10-HDA 2,5 g/100 ml: c = 12,5 µg/ml (corresponds to 2,5 g/100 g in sample)	Pipette (x * 963) µl 10-HDA-S_0 standard stock solution into a 10 ml measuring flask and dilute to volume with sample solvent

x = factor of 10-HDA-S_0 stock solution (see B.1.1.3)

B.1.3.4 Sample determination

Inject 20 µl into the chromatograph.

Measure filtered (diluted) extract by HPLC at 216 nm against an external standard calibration curve.

B.1.4 Calculation

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:

$$y = ax + b \tag{B.1}$$

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 10-HDA in the measuring solution from the calibration curve as follows:

$$x' = (y' - b) / a \tag{B.2}$$

where

x' is the concentration [µg/ml] of 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 ($C_{10\text{-HDA}}$) in royal jelly (sample in g/100 g) is given by Formula (B.3):

$$C_{10\text{-HDA}} = x' \times 40/m \tag{B.3}$$

where

x' is the calculated concentration [µg/ml] of 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 (1ml) and the volume of the measuring flask used for dilution (10 ml);

m is the actual mass of the royal jelly sample, in mg.

B.1.5 Precision

Relative deviation of parallel experiments shall not be more than 2,0 % (lyophylisates) and 5,0 % (fresh royal jelly).

B.2 HPLC-UV internal standard (Alternative method)

B.2.1 Reagents

Use only double distilled water.

B.2.1.1 Methanol, UV purity or analytical purity with light transmittance above 30 % at detection wavelength (210 nm).

B.2.1.2 Anhydrous alcohol, GR.

B.2.1.3 Internal standard substance, trans-2-hexenoic acid, of 99,0 % purity.

B.2.1.4 10-HDA standard, of purity above 99,0 %. Decompress and dry for 24 h in the vacuum drying oven or desiccator with concentrated sulfuric acid before it is used.

B.2.1.5 10-HDA standard solution. Weigh approximately 12,5 mg dried 10-HDA standard sample, weigh accurately, dissolve with anhydrous alcohol and transfer it to a 25 ml volumetric flask, dilute to the mark with anhydrous alcohol and mix evenly.

The concentration of 10-HDA obtained in solution is 0,5 mg/ml.

B.2.1.6 Internal standard solution. Weigh approximately 650 mg of trans-2-hexenoic acid, weigh accurately, dissolve with anhydrous alcohol and transfer it to a 1 000 ml volumetric flask, dilute to the mark with anhydrous alcohol and mix evenly.

The concentration of internal standard solution obtained in solution is 0,65 mg/ml.

B.2.1.7 Hydrochloric acid (c = 0,03 mol/l). Take 100 ml of 0,1 mol/l hydrochloric acid, add 200 ml double distilled water.

B.2.1.8 Mobile phase, Φ (CH_3OH + 0,03 mol/l HCl+H_2O) = 55 + 10 + 35 or (CH_3OH + 25 mmol/l phosphate buffer pH 2,5) = 55 + 45.

B.2.2 Apparatus

B.2.2.1 HPLC, with ultraviolet detector, recorder or microprocessor.

B.2.2.2 Chromatographic column, 4,6 mm × 250 mm stainless steel, fill amorphous silica gel with C18 bonded stationary phase, of 5 μm or 10 μm particle size.

B.2.2.3 Ultrasonic cleaner.

B.2.2.4 Mixer, Vortex mixer or equivalent.

B.2.2.5 Analytical balance, capable of weighing to the nearest 0,000 01 g.

B.2.3 Procedure

B.2.3.1 Sample treatment

Defreeze the sample to the room temperature and stir evenly with glass rod, weigh approximately 0,5 g, and put it in a 50 ml volumetric flask that has been weighed already, weigh accurately, add 1 ml of 0,03 mol/l hydrochloric acid and 2 ml water, put it on the vortex mixer and mix to dissolve the sample,

add anhydrous alcohol 30 ml add while shaking lightly, add 10 ml internal standard solution accurately, dilute to the mark with anhydrous alcohol and mix evenly, put it in the ultrasonic bath for 15 min immediately, or put it on the vortex mixer and shake for 15 min, take out, test after centrifugation for 10 min at 3 000 r/min and filtration with 0,45 μm membrane filter, if necessary. Put it in a refrigerator if it shall not be tested immediately.

B.2.3.2 Chromatography condition

Test wavelength: 210 nm; column temperature: 35 °C; mobile phase velocity of flow: 1 ml/min.

B.2.3.3 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 10 ml volumetric flasks. Add accurately 2 ml internal standard solution, dilute to the mark with anhydrous alcohol, and mix evenly. Weigh respectively 10 μl of these solutions, inject it into the chromatograph, plot the mass ratio of 10-HDA per internal standard against the peak area ratio of that, and draw a linear calibration curve. Calculate the correction factor, *F*, which is the slope of the calibration curve.

B.2.3.4 Sample determination

Weigh 10 μl sample solution, inject it into the chromatograph, and correct the measurement by "internal standard method".

B.2.4 Calculation

The 10-HDA content in royal jelly, is given by Formula (B.4):

$$X_2 = F \times \frac{A_i}{A_s} \times \frac{m_s}{m_i} \times 100 \tag{B.4}$$

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 peak area of internal standard in sample;

m_s is the mass of the internal standard, in grams;

m_i is the mass of sample, in grams.

B.2.5 Precision

Relative deviation of parallel experiments shall not be more than 2,0 %.

Annex C
(normative)

Determination of protein

C.1 Kjeldahl method (automatic) (Reference method)

C.1.1 Reagents

C.1.1.1 Concentrated sulfuric acid, 95 % to 98 %, reagent grade.

C.1.1.2 Catalyst. Weigh 7,0 g potassium sulfate and 0,4 g copper sulfate.

C.1.1.3 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.

C.1.1.4 Boric acid solution, 4 % (w/v). Dissolve 400 g boric acid in 5 to 6 l hot deionized water. Mix and add more hot deionized 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/l NaOH or 0,1 mol/l HCl. Or 25 ml Sher mixed indicator and dilute to a final volume.

C.1.1.5 Sodium hydroxide solution. Weigh 32 g sodium hydroxide, dilute to 100 ml with distilled water.

C.1.1.6 Hydrochloric acid standard solution, 0,1 mol/l.

C.1.2 Apparatus

C.1.2.1 Analytical balance, capable of weighing to the nearest 0,000 1 g.

C.1.2.2 Digestion block. Aluminium alloy block with adjustable temperature device for measuring and controlling block temperature (Tecator Digestion System 20, 1015 Digestor[2)] or KjelDigester K-449[2)], SpeedDigester K-439[2)] or equivalent).

C.1.2.3 Digestion tubes, 250 ml to 300 ml.

C.1.2.4 Distillation units, Foss Tecator 2200[2)], BUCHI KjelMaster K-375[2)] or equivalent, to accept 250 ml to 300 ml.

C.1.2.5 Titration flask, 500 ml graduated Erlenmeyer flask (for collection and titration of distillate).

C.1.2.6 Fume exhaust manifold, with polytetrafluoroethylene (PTFE) ring seals, connected to a water aspirator in a hooded sink.

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2) Example of a suitable product available commercially. This information is given for the convenience of users of this document and does not constitute an endorsement by ISO of this product.

C.1.2.7 Nitrogen free weighing boats.

C.2.1.8 Pipetting dispenser, –25 ml, adjustable volume, attached to a 2,4 l acid bottle.

C.1.3 Procedure

C.1.3.1 Digestion

Weigh approximately 1 g of royal jelly sample onto a tared, N free weighing boat. Fold paper around material and drop into a Kjeldahl tube.

Add the catalyst, add 12 ml of sulfuric acid, using pipetting dispenser. Hold the mixture overnight.

Place fume manifold tightly on tubes, and turn water aspirator on completely. Place rack of tubes in preheated block (at 420 °C). After 10 min, and 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 vacuum source to prevent loss of sulfuric acid. Digest additional 50 min. Total digestion time is approximately 60 min.

Let tubes cool. Add deionized water to each tube to a total volume of approximately 80 ml.

C.1.3.2 Distillation

Place 32 % NaOH in alkali tank of distillation unit. Adjust volume dispensed to 50 ml. Attach digestion tube containing diluted digest to distillation unit, or use automatic dilution feature. 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. Steam distil until ≥150 ml distillate is collected; remove receiving flask.

C.1.3.3 Titration

Titrate H_3BO_3 receiving solution with standard 0,1 mol/l HCl to violet or grey end point. Record millilitres of HCl to at least the nearest 0,05 ml.

C.1.4 Calculation

The protein content in royal jelly is given by Formula (C.1):

$$N = \frac{\left(V_s - V_b\right) \times M \times 14{,}01}{m \times 10} \times 6{,}25 \qquad \text{(C.1)}$$

where

N is the protein content in royal jelly, given by mass fraction, %;

V_s is the volume of standardized acid consumed when the sample is titrated, in millilitres;

V_b is the volume of standardized acid consumed when blank titration is made, in millilitres;

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.

C.2 Kjheldal method (classical)

C.2.1 Reagents

C.2.1.1 Concentrated sulfuric acid, w = 95 %~98 %.

C.2.1.2 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.

C.2.1.3 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 Sher mixed indicator.

C.2.1.4 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.

C.2.1.5 Sodium hydroxide solution (ρ = 400 g/l). Weigh 32 g sodium hydroxide, and dilute to 100 ml with distilled water.

C.2.1.6 Dilute sulfuric acid. Using a pipette, take 5,7 ml concentrated sulfuric acid, and dilute to 100 ml with distilled water.

C.2.1.7 Hydrochloric acid standard solution (0,1 mol/l). Dilute to 10 times before using.

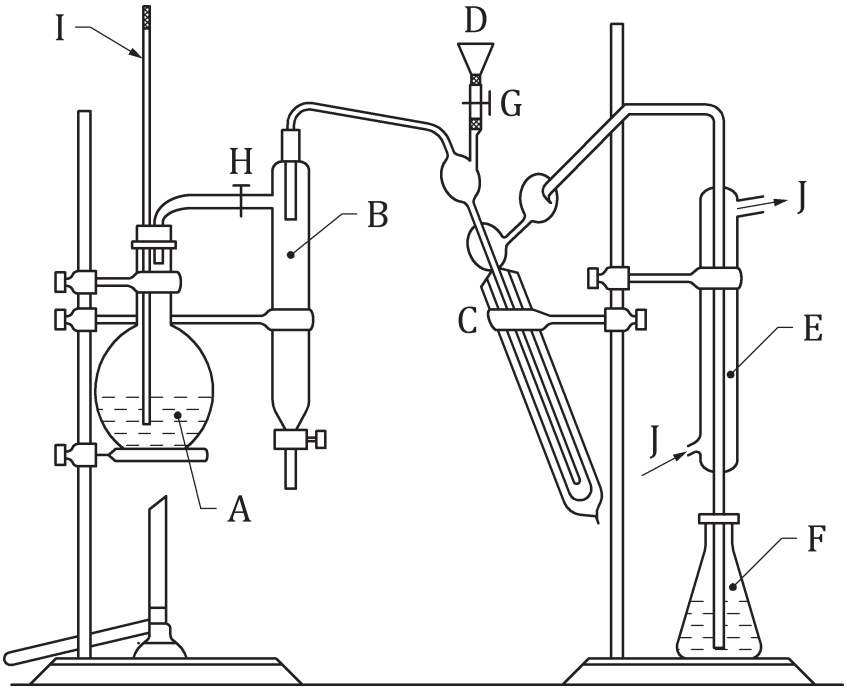
C.2.2 Apparatus

C.2.2.1 Kjeldahl nitrogen determination method digestion equipment, 50 ml Kjeldahl flask (if far infrared digesting electric furnace is used, a 50 ml digesting tube and retort funnel shall be collocated).

C.2.2.2 Acid burette, 10 ml.

C.2.2.3 Analytical balance, capable of weighing to the nearest 0,000 01 g.

C.2.2.4 Semimicro method distillation unit (see Figure C.1).



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		

Figure C.1 — Semimicro method distilled unit for determining protein content

C.2.3 Procedure

C.2.3.1 Cleaning of distillation unit

Link distillation unit, add proper amount of distilled water and a few drops of methyl red indicator in bottle A, add dilute sulfuric acid to make it acidic, add a few granules of glass beads and zeolites, add 50 ml distilled water from funnel D, close nip G, open condensate water, and boil the distilled water in bottle A. When the vapour 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. Open nip G, discharge the distilled water in bottle B, and close nip B and G. 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. Clean the apparatus twice or three times like this.

C.2.3.2 Digestion

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 a Kjeldahl flask or a digesting tube. 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, 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. 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, transfer to a 100 ml volumetric flask after it is cooled, dilute to the mark with distilled water and shake evenly for later use.

C.2.3.3 Distillation

Weigh 10 ml boric acid of 20 g/l, put it in a 100 ml conical flask, add five drops of mixed indicator, immerge the top of the condenser tube in the solution, 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, clean the funnel D repeated with a little distilled water, close nip G and add a few millilitres of distilled water in funnel D for the purpose of closing tube. Heat bottle A (dilute sulfuric acid shall be added drop by drop into the distilled water in the bottle so as to keep its acidity) and distil the vapour. 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 vapour continue to wash for 1 min, drip-washing the top with a little distilled water and stop distillation.

C.2.3.4 Titration

The absorption solution shall be titrated with 0,01 mol/l hydrochloric acid standard solution. When the colour changes from cyan to grey purple, the end point has been reached.

C.2.4 Calculation

The protein content in royal jelly is given by Formula (C.2):

$$X_3 = \frac{\left(V_1 - V_0\right) \times c_1 \times 0{,}014}{m_4 \times 5 / 100} \times 6{,}25 \times 100 \qquad \text{(C.2)}$$

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 standard 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.

C.2.5 Precision

Relative deviation of parallel experiments shall not be more than 3,0 %.

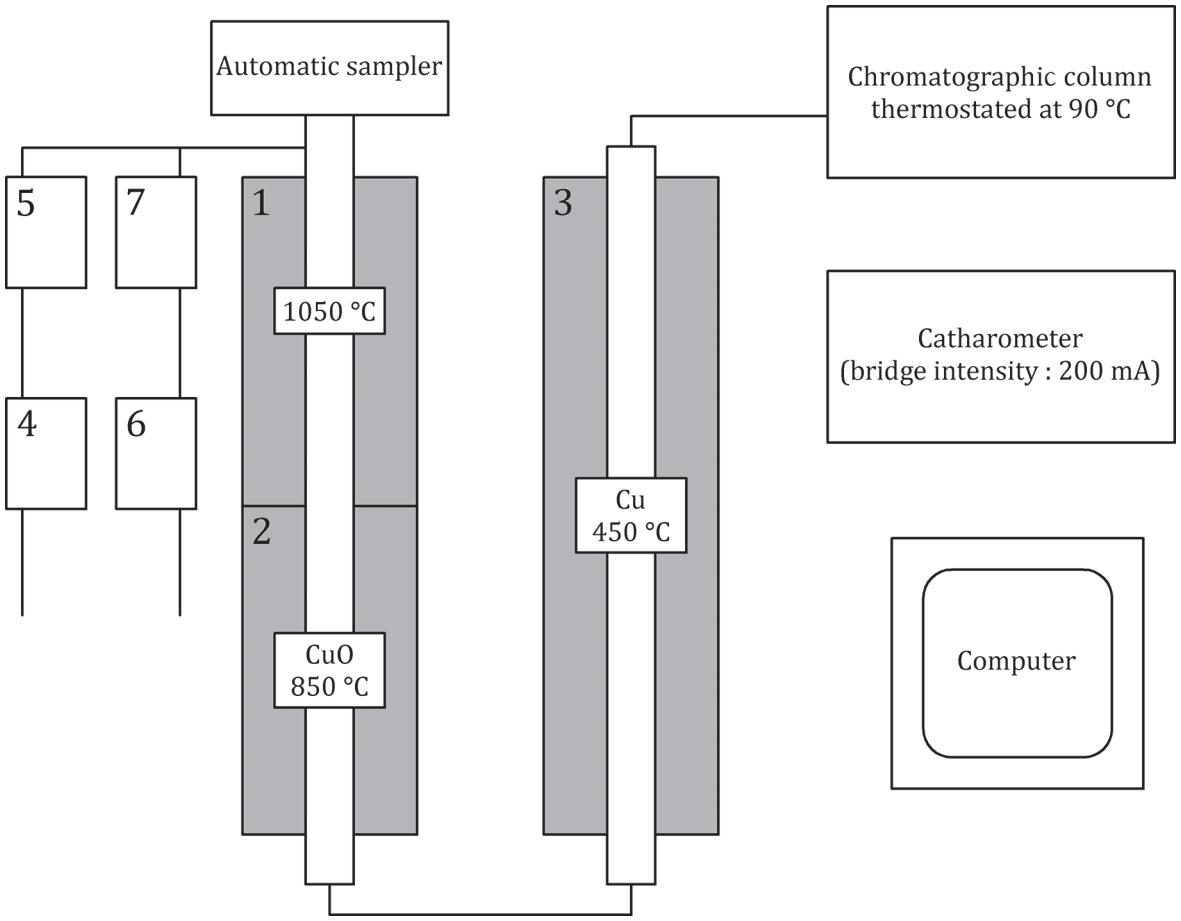
C.3 Elemental analyser

C.3.1 Apparatus

C.3.1.1 Analytical balance, capable of weighing to the nearest 0,000 01 g

C.3.1.2 Elemental analyser.

See Figure C.2.



Key

1 combustion unit
2 post-combustion unit
3 reduction furnace
4 helium (at about 1,5 bars)
5 helium mass flow regulator (at 50 ml/min)
6 oxygen pressure regulator (at about 1,5 bars)
7 oxygen mass flow regulator (at 2 ml/min)

Figure C.2 — Elemental analyser unit

C.3.2 Procedure for determination of the nitrogen content

Exactly weigh approximately 2 mg of lyophilised royal jelly in a silver capsule. Introduce it in a combustion unit hold at 1 050 °C. A helium stream containing 5 % of oxygen carries the gases derived from the combustion on a post-combustion unit filled with copper oxide and maintained at 850 °C. Nitrogen is transformed in nitrogen oxide and (NOx) are reduced to N_2 in a copper reduction furnace hold at 450 °C.

Water is trapped with anhydrous magnesium perchlorate.

N_2 is separated from CO_2 and water using a gas chromatographic column (Hayesep Q 60/80 mesh, Supelco[3)], 3,5 m × 0,63 cm) hold at 45 °C. The nitrogen content is then quantified with a catharometer (Wheatstone bridge intensity: 200 mA).

3) Example of a suitable product available commercially. This information is given for the convenience of users of this document and does not constitute an endorsement by ISO of this product.

C.3.3 Calibration

A calibration of the analyser is made every day with standards with a purity superior to 99,5 %.

C.3.4 Precision

Results of parallel experiments shall not differ from more than 0,3 %.

C.3.5 Calculation

The nitrogen content (%N) of royal jelly is obtained using Formula (C.3):

$$\%\mathrm{N} = \frac{m_{\mathrm{nitrogen}}}{m_{\mathrm{total}}} \times 100 \qquad \text{(C.3)}$$

where

%N is the nitrogen content;

m_{nitrogen} is the mass of nitrogen obtained from the calibration curve, in µg;

m_{total} is the mass of royal jelly, in µg.

The protein content of royal jelly is given by Formula (C.4):

$$\text{protein content} = 6{,}25 \times \%\mathrm{N} \qquad \text{(C.4)}$$

where

%N is the nitrogen content;

6,25 is a multiplicative factor corresponding to the reverse nitrogen content of a protein.

Annex D
(normative)

Determination of sugar

D.1 Liquid chromatographic analysis (Reference method)

D.1.1 Reagents

D.1.1.1 Acetonitrile, HPLC quality.

D.1.1.2 Methanol, HPLC quality.

D.1.1.3 Ultrapure water.

D.1.1.4 Sugar standards, purity superior or equal to 98,0 %.

D.1.2 Apparatus

D.1.2.1 LC with refractive index detector, recorder or microprocessor.

D.1.2.2 HPLC column, with amino-modified phase.

D.1.2.3 Ultrasonic bath.

D.1.2.4 Centrifuge.

D.1.2.5 Analytical balance, capable of weighing to the nearest 0,000 1 g.

D.1.3 Procedure

D.1.3.1 Sample treatment

Weigh accurately approximately 2 g of royal jelly in a beaker. Add some millilitres of a solution MeOH/H_2O:75/25 under magnetic stirring. Transfer in a 20 ml volumetric flask and complete with the same solution MeOH/H_2O. Centrifuge 10 min at 4 000 rpm. Filter the supernatant before chromatographic injection.

D.1.3.2 Chromatography conditions

Mobile phase: acetonitrile: water (75: 25)

Flow: 1 ml/min

Column temperature: 30 °C

D.1.3.3 Calibration

Standard (M): weigh exactly the sugar standard in order to obtain in anhydrous sugar a concentration of 1 g /100 ml. Transfer in a 100 ml flask. Add around 25 ml of water and stir.

Complete to the mark with methanol.

F1: Dilute 10 ml of solution M in a 20 ml volumetric flask with a mixture MeOH/H_2O:75/25

F2: Dilute 5 ml of solution M in a 20 ml volumetric flask with a mixture MeOH/H_2O:75/25

D.1.4 Calculation

The concentration of the sugar *i* in sample is calculated using Formula (D.1):

$$C_i = k_i \times A_i \quad \text{(D.1)}$$

where

C_i is the concentration of the sugar *i* in sample; in mg/ml;

k_i is the response factor of sugar *i*, which is calculated from the slope of calibration curve constructed by the area against concentration of the standard solutions (M, F1, F2);

A_i is the area of sugar *i* in sample.

Total sugar in royal jelly is calculated using Formula (D.2):

$$\%\text{Sugar } i = C_i \times 20/m \times 100 \quad \text{(D.2)}$$

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 sample, in mg.

%Total sugar = %Sugar (fructose + glucose + sucrose)

D.1.5 Precision

Relative deviation of parallel experiments shall not be more than 3,0 %.

D.2 Titration method

D.2.1 Reagents

D.2.1.1 Glucose standard solution. Weigh accurately 1,000 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 1 000 ml with distilled water.

Every millilitre of this solution equals to 1 mg glucose

D.2.1.2 Alkaline cupric tartrate TS solution A. Dissolve 15 g copper sulfate ($CuSO_4 \cdot 5H_2O$) and 0,05 g methylene blue, in 1 000 ml water, and store in a tightly stoppered bottle.

D.2.1.3 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 1 000 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.

D.2.1.4 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.

D.2.1.5 Potassium ferrocyanide, ρ = 106 g/l.

D.2.1.6 Concentrated hydrochloric acid, w = 36 % ~ 38 %.

D.2.1.7 Hydrochloric acid, c = 6 mol/l. Weigh 50 ml hydrochloric acid, add distilled water and dilute to 100 ml.

D.2.1.8 Sodium hydroxide solution, ρ = 200 g/l.

D.2.1.9 Methyl red indicator, ρ = 1 g/l, ethanol solution.

D.2.2 Apparatus

D.2.2.1 Electric-heated thermostatic water bath, temperature fluctuation ±1 °C.

D.2.2.2 Analytical balance, capable of weighing to the nearest 0,000 1 g, or **electronic balance**, capable of weighing to the nearest 0,000 1 g.

D.2.3 Procedure

D.2.3.1 Sample treatment

Weight approximately 4 g of royal jelly sample, put it in a 100 ml volumetric flask, add 50 ml distilled water, shake till dissolution of the sample, then add 5 ml zinc acetate solution and potassium sodium tartrate respectively and slowly, dilute to the mark with distilled water, and mix evenly. Allow to stand for 30 min and filtrate with dried filter paper, discard a few millilitres of initial filtrate. The filtrate is for later use.

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 (ρ = 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.

D.2.3.2 Sample solution titration

Take accurately 5 ml of alkaline cupric tartrate TS solution A and B respectively, 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, add sample solution drop by drop from the burette and keep the solution in boiling state. When the solution colour starts to lose, titrate at the speed of one drop per 2 s. The end point is reached when the colour blue has just faded. Record the volume of the sample solution consumed.

D.2.4 Calculation

The total sugar content in royal jelly is given by Formula (D.3):

$$X_4 = \frac{T}{m_5 \times V_2 \,/\, 100 \times 1 \,/\, 2 \times 1\,000} \times 100 \qquad \text{(D.3)}$$

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 millilitres.

D.2.5 Precision

Relative deviation of parallel experiments shall not be more than 3,0 %.

D.3 Gas chromatographic analysis

D.3.1 Reagents

D.3.1.1 Hexamethyldisilazane, puriss ≥99 %.

D.3.1.2 Trimethylchlorosilane, puriss ≥99 %.

D.3.1.3 Pyridine, puriss ≥99,8 %.

Anhydrous pyridine is obtained by distillation over calcium hydride.

D.3.1.4 Sorbitol (internal standard), puriss ≥99 %.

D.3.2 Apparatus

D.3.2.1 GC with flame ionization detector, recorder or microprocessor.

D.3.2.2 Chromatographic column, HP5-MS column (30 m × 0,25 mm; 0,25 µm).

D.3.2.3 Analytical or **electronic balance**, capable of weighing to the nearest 0,000 01 g.

D.3.3 Procedure

D.3.3.1 Sample treatment

Weigh accurately about 40 mg of lyophilized royal jelly and 1 mg of sorbitol, introduce them in a glass reactor. Close tightly. Then, add 1 ml of anhydrous pyridine. Stir the mixture for 5 min with the reactor sealed. Then add 200 µl of hexamethyldisilazane and stir the mixture for 5 min. Add 100 µl of trimethylchlorosilane. Stir for 30 min. Leave the mixture for 20 h at room temperature with the reactor sealed.

D.3.3.2 Chromatography conditions

D.3.3.2.1 Helium as carrier gas (5,0 grade).

Constant pressure 22 psi.

D.3.3.2.2 Injection volume: 2 µl.

D.3.3.2.3 Injector and detector temperatures set at 280 °C.

D.3.3.2.4 Program of oven temperature: Maintain initial temperature (150 °C) for 5 min, then increase to 325 °C at a rate of 3 °C/min.

Maintain the final temperature for 10 min.

D.3.3.3 Sugar identification

Use reference standards or retention indices to identify the different sugars. Determine the retention indices of each sugar by injecting the standard with the same analytical and chromatographic conditions.

Inject a mixture of paraffins from C15 to C40 prior to each batch of samples.

D.3.3.4 Sugar quantification - Determination of correction factor

Carry out sugar quantification by internal calibration with sorbitol as internal standard. A response factor, or mass correction factor, is calculated for each sugar using Formula (D.4):

$$k_i = \frac{A_{SI}}{A_i} \times \frac{M_i}{M_{SI}} \tag{D.4}$$

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*.

For compounds that are present within two anomeric forms, calculate the area by adding the area of both anomers.

D.3.3.5 Calculation

The mass of the sugar *i* in the royal jelly sample is calculated using Formula (D.5):

$$m_i = k_i \times \frac{A_i}{A_{SI}} \times m_{SI} \tag{D.5}$$

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 sample is calculated using Formula (D.6):

$$\%\text{sugar}_i = \%\text{MS} \times \frac{m_i}{m_{\text{sample}}} \quad \text{(D.6)}$$

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.

D.3.4 Precision

Relative deviation of parallel experiments shall not be more than 3,0 %.

Annex E
(normative)

Determination of total acidity

E.1 Reagents

E.1.1 Sodium hydroxide, c = 0,1 mol/l.

E.2 Apparatus

E.2.1 pH-meter, pH value, to the nearest 0,1.

E.2.2 Burette, 10 ml.

E.2.3 Analytical balance, capable of weighing to the nearest 0,000 1 g.

E.3 Procedure

Weigh 1,00 g royal jelly sample, put it in a 100 ml beaker, and add 75 ml boiled and cooled distilled water, titrate with sodium hydroxide standard solution(c = 0,1 mol/l). The end point is achieved when the pH-meter indicates at pH 8,3.

E.4 Calculation

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.

The acidity in royal jelly is given by Formula (E.1):

$$\text{Acidity [(1 mol/l NaOH) ml/100g]} = (V \times c \times 100)/m \qquad \text{(E.1)}$$

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.

E.5 Precision

Relative deviation of parallel experiments shall not be more than 5,0 %.

Annex F
(normative)

Determination of total lipid

F.1 Reagents

F.1.1 Diethyl ether, of purity above 99,5 %. Or use *tert*-buthylmethyl ether (MTBE) as alternative extraction solvent.

F.1.2 Celite.

F.2 Apparatus

F.2.1 Soxhlet extraction apparatus, with soxhlet extraction tube (internal diameter ca. 40 mm), extraction bottle and condenser tube.

F.2.2 Thimble filter, of internal diameter 25 mm to 30 mm, of length 100 mm to 120 mm.

F.2.3 Thermostatic bath.

F.2.4 Drying oven.

F.2.5 Vacuum drying oven.

F.3 Procedure

Weigh accurately approximately 2,5 g of royal jelly sample in a beaker and add 3 g to 5 g of Celite. Mix the sample and Celite well with a glass rod until the mixture is equalized. 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, put the defatted cotton into the upper half of thimble filter. Dry in air the thimble filter until the smell of diethyl ether has gone. Dry the thimble filter for 2 h at 70 °C under the pressure in vacuum drying oven. Add 100 ml to 150 ml diethyl ether into an extraction bottle which is dried until a constant weight, put the thimble filter into an extraction tube, and connect the extraction tube to a condenser tube and the extraction bottle. Extract lipid on a thermostatic bath at approximately 50 °C for 8 h. 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. Wipe the outside of the extraction bottle, dry it in a drying oven at 105 °C for 1 h and weigh it after cooling in a desiccator for 1 h.

F.4 Calculation

The total lipid in royal jelly is given by Formula (F.1):

$$X_5 = \frac{m_7 - m_6}{m_8} \times 100 \qquad \text{(F.1)}$$

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 weight, 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.

Annex G
(normative)

Determination of C13 isotopic ratio

G.1 General

See Reference [2].

G.2 Procedure

100 to 1 000 µg of royal jelly are loaded into a tin (or silver) capsule.

Samples are dropped from a carousel-type autosampler into a reactor filled with chromium oxide and cobaltous/cobaltic oxide.

Automated oxygen dosing ensures complete combustion of the sample. Subsequent to combustion, NOx compounds are reduced to N_2 in a reactor filled with reduced copper.

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.

G.3 Calculation

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.

Annex H
(informative)

Determination of furosine

H.1 General

See Reference [3].

H.2 Reagents

Use only ultrapure water.

H.2.1 Methanol for HPLC.

H.2.2 Hydrochloric acid, HCl, 8 mol/l.

H.2.3 Hydrochloric acid, HCl, 3 mol/l.

H.2.4 Sodium acetate.

H.2.5 Glacial acetic acid.

H.2.6 Furosine reference standard (Neosystem Laboratoire-Strasbourg, France[4)] or equivalent).

H.3 Apparatus

H.3.1 Analytical balance, capable of weighing to the nearest 0,000 01 g.

H.3.2 HPLC with binary pump system and **ultraviolet detector** (DAD in preferred), **recorder** or **microprocessor**.

H.3.3 Chromatographic column, reversed phase C-8,25 cm × 4,6 mm (5 µm particles) or equivalent.

H.3.4 SPE cartridge, C18, 500 mg (SPE-PAK cartridge from Waters Corp., Milford, MA[4)] or equivalent).

H.3.5 Syringe-tip filter, 0,45 µm, [membrane: polytetrafluoroethylene (PTFE) or equivalent].

H.3.6 Vial, amber glass vial.

H.3.7 Vial, glass vial for hydrolysis with polytetrafluoroethylene (PTFE) seal (Duran[4)] or equivalent).

H.4 Chromatography conditions

Mobile phase: 0,06 mol/l sodium acetate, pH 4,3 acetic acid.

4) Example of a suitable product available commercially. This information is given for the convenience of users of this document and does not constitute an endorsement by ISO of this product.

Flow: 2 ml/min.

Column temperature: 30 °C.

Detector: UV-VIS: 280 nm.

Injection volume: 20 µl to 50 µl.

H.5 Procedure

An aliquot of sample (0,35 g), corresponding to about 30 mg to 70 mg of protein, is hydrolyzed with 8 ml of 8 mol/l HCl at 110 °C for 23 h. After hydrolysis, 0,5 ml of the hydrolysate is purified on a SPE C18 cartridge (SPE procedure: conditioning the SPE cartridge with 5 ml MeOH, followed with 10 ml ultrapure water, sample application: 0,5 ml Hydrolyzate sample, elute by gravity) (discharge the eluate and do not dry the cartridge); elute with 1 ml × 4 of HCl 3 mol/l, dry the cartridge in air, collect all eluate in a 5 ml volumetric amber glass fill at 5 ml with HCl 3 mol/l solution. Filter with syringe-tip 0,45 µm filter in amber glass vial, store at −20 °C until the HPLC analysis.

Inject 50 µl in a HPLC system.

Protein determination: proceeded as Annex C.

H.6 Calculation

Quantificate the furosine by external calibration standard, and express the value as:

Furosine = mg Furosine/100 g protein

Annex I
(informative)

Pollen screening

I.1 Method including pollen acetolysis

I.1.1 Samples preparation

Weigh 2,0 g of royal jelly into a pointed centrifuge tube (capacity 50 ml).

Add 10 ml of distilled water and mix thoroughly.

Add 8 ml of NaOH 32 % and mix thoroughly.

Fill the tube with distilled water up to 45 ml.

When the mix is clear, stop the reaction with 5 ml of ethanol.

Centrifuge at 4 000 r/min for 10 min. Remove the supernatant liquid.

Add 45 ml of distilled water and mix thoroughly.

Centrifuge at 4 000 r/min for 10 min. Remove the supernatant liquid.

Add 15 ml of glacial acetic acid 100 %, mix and centrifuge.

Remove the supernatant liquid.

I.1.2 Procedure: acetolysis of pollens

Mix acetic anhydride and sulfuric acid 96 % (9/1 v/v).

Use dry materials because this mix reacts vigorously with water.

Add 1 ml of this mix into the centrifuge tube. Mix thoroughly.

Place the tube in an 80 °C water bath for 15 min.

Add 30 ml of acetic acid to stop the acetolytic reaction and mix.

Centrifuge at 4 000 r/min for 10 min. Remove the supernatant liquid.

Add 35 ml of distilled water. Mix thoroughly.

Centrifuge at 4 000 r/min for 10 min. Remove the supernatant liquid.

Put the tube upside down on absorbent paper and let dry.

Add 50 µl of mounting liquid (glycerol 90 %/formic aldehyde 40 %, 9/1, v/v).

Draw on a slide, a first rectangle with glue (ex: histolaque). Allow to dry.

Dispense the suspension onto the slide.

Draw a second rectangle with glue around the first one and cover with a cover slip.

I.1.3 Identification and counting

Pollens are identified with keys, reference slides and photos atlas.

Examples are provided in References [4], [5] and [6].

Count at least 100 pollens with G × 1 000. Scan the slide horizontally because of the poor pollinic density.

Calculate the total of pollens and the relative amount of each one.

I.2 Method without pollen acetolysis

I.2.1 References

The method is based on References [7] and [8] and on the DIN 10760 method.[9] During the entire procedure, great care shall be taken to prevent any contamination from foreign pollen coming from either previous preparations (disposable supplies are recommended as far as possible) or from airborne grains (close windows and limit the exposure).

I.2.2 Sample preparation

— Weigh 1,0 g of royal jelly into a pointed centrifuge tube (capacity ca. 50 ml) and add 10 ml of KOH 1 %.

— Dissolve the royal jelly by mixing thoroughly (vortex).

— Centrifuge for 10 min at 1 000*g*.

— Suck up the liquid supernatant by means of a Pasteur pipette connected to a pump.

— Add 10 ml of distilled water and dissolve the royal jelly by mixing thoroughly (vortex); fill the tube with water up to 45 ml.

— Centrifuge again for 10 min at 1 000*g*.

— Decant the supernatant liquid, allowing any excess liquid to be taken up on absorbent paper.

— Heat a heating plate to 40 °C and liquefy the glycerine gelatine (mounting medium).

— Draw a square on the back of the microscope slide and put the microscope slide onto the heating plate.

— Transfer the entire sediment onto the slide with a single use Pasteur pipette and spread it evenly over the marked area. Leave the slide on the heating plate only for the time strictly necessary to dry the sediment.

— Apply one drop of glycerine gelatine on a cover slip forming a large cross diagonally and place the cover slip on the slide.

I.2.3 Identification and counting

Count pollen grains under the microscope following the matrix represented in Figure I.1. Pollen grains are counted along five parallel equidistant lines uniformly distributed from one edge of the 10 mm × 10 mm smear to the other (in total at least 500 pollen grains are counted). See Reference [7] for further details.

Abortive, irregular or broken pollen grains are counted if they can be identified. Note separately non-identifiable or non-identified grains. For each pollen type, calculate the relative frequency as a percentage of the total number of pollen grains counted.

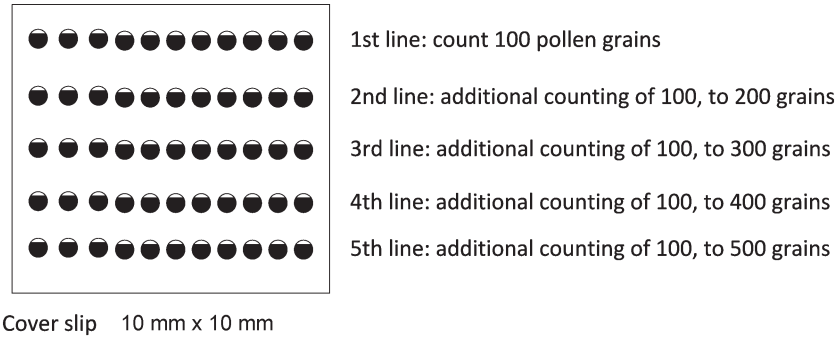


Figure I.1 — Matrix, used for counting pollen grains, which guarantees representative results (= a whole microscopic field of view)[7]

Bibliography

[1] Abeille de France (1990), n° 753, p.405

[2] AOAC 998: 12 for honey

[3] J. Agric. Food Chem. 2002, **50** pp. 2825–2829

[4] Pollen analysis, Moore / Webb / Collinson, Second Edition

[5] Atlas photographiques de Maurice Reille: Pollens et Spores d'Europe et d'Afrique du Nord

[6] Pollen flora of China, Second Edition

[7] Ricciardelli D'Albore G., & Battaglini M. Origine géographique de la gelée royale. Apidologie (Celle). 1978, **9** pp. 1–17

[8] Von Der Ohe W., Persano Oddo L., Piana M. L., Morlot M., Martin P. Harmonized methods of melissopalynology, Apidologie 35 (2004), suppl. 1, 18-25

[9] DIN 10760:2002, *Determination of the relative pollen content of honey*

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