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Bee pollen — Specifications

Pollen en pelotes — Spécifications

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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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This document was prepared by Technical Committee ISO/TC 34, *Food products*, Subcommittee SC 19, *Bee products*.

Any feedback or questions on this document should be directed to the user's national standards body. A complete listing of these bodies can be found at www.iso.org/members.html.

Introduction

Pollen is the male gametes of seed plant flowers (angiosperms and gymnosperms). Angiosperm pollen is formed on the stamens as tiny grains. It is a coarse, powdery substance used for transferring haploid male genetic material from the anther of a single flower to the stigma of another in cross-pollination. Commercial bee pollen is mainly the pollen collected by the honey bee (*Apis* spp.) to feed its larvae in the early stages of development.

Collected flower pollen is accumulated as orbicular pellets in pollen baskets on the rear legs of the honey bee. When visiting flowers, bees touch the stamens, moisten the pollen with nectar and salivary substances, and use their hind legs to compress and agglutinate the pollen into the pollen baskets. When the bees return to the hive, the bee pollen can be harvested at the hive entrance using a specific trap.

Bee pollen is the bees' primary source of essential nutrients: proteins, carbohydrates, lipids, crude fibre, minerals and other substances in minor concentrations, namely vitamins, carotenoids and flavonoids. Bee pollen is composed of all essential amino acids. Thus, bee pollen is harvested as a nutrient-rich food and tonic for humans, with various healthcare function claims. In addition, bee pollen is a raw material from which bees produce bee bread, mixed with honey and bee saliva stored in brood cells, and used as a fermented product rich in proteins and lipids in the bee diet.

This document clarifies the definition of various types of bee pollen (dried, frozen and lyophilized), establishes their composition indicators and test methods, specifies the storage, packaging, transportation and labelling requirements, and provides quality standards for the international bee pollen trade. It is a matter for the parties concerned whether to apply the requirements of this document to a consignment or a lot of bee pollen. The primary supply of protein and vitamins is necessary for the secretion of royal jelly during the larval breeding stage of the beehive. Although the bee pollen source presents a high variability, which results from the various local floral sources in the different countries, the relatively similar and stable characteristics of nutrients do not affect a beehive's nutritional requirements.

Bee pollen — Specifications

1 Scope

This document specifies the quality requirements, analytical methods, and packaging, labelling, marking, storage and transportation conditions for bee pollen.

This document is applicable to bee pollen collected at the entrance of beehives by *Apis mellifera* and other bees' species of colonies, as well as dried, frozen and lyophilized bee pollen.

It does not apply to crushed powdery bee pollen and products made from bee pollen.

2 Normative references

The following documents are referred to in the text in such a way that some or all of their content constitutes requirements of this document. For dated references, only the edition cited applies. For undated references, the latest edition of the referenced document (including any amendments) applies.

ISO 3696, *Water for analytical laboratory use — Specification and test methods*

ISO 22005, *Traceability in the feed and food chain — General principles and basic requirements for system design and implementation*

3 Terms and definitions

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

ISO and IEC maintain terminology databases for use in standardization at the following addresses:

— ISO Online browsing platform: available at https://www.iso.org/obp

— IEC Electropedia: available at https://www.electropedia.org/

3.1
pollen
male gametophytes produced in the anther of the stamen of flower structures in plants (predominantly angiosperms), which appears as a fine to coarse powder of microscopic grains carried by insects or the wind

3.2
worker bee
female bee with underdeveloped reproductive abilities that is responsible for different duties, such as foraging, nursing, safeguarding and cleaning, within the colony of honey bees

3.3
bee pollen
result of the agglutination of flower *pollen* (3.1) grains with nectar and salivary substances and collected by *worker bees* (3.2)

Note 1 to entry: Bee pollens are collected at the hive entrance.

Note 2 to entry: Bee pollen is a raw material from which bees produce bee bread.

3.4
monofloral bee pollen

bee pollen (3.3) where the percentage of *pollen* (3.1) is linked to the size of the pollen grains

Note 1 to entry: After analysis of the pollens, the size of the pollen grains suspected of being dominant in the sample shall be checked to see if the percentage by volume is at least 80 % of the total volume of pollen present.

3.5
multifloral bee pollen

bee pollen (3.3) collected by *worker bees* (3.2) from different flowers but in an insufficient quantity that none of the *pollen* (3.1) reaches the percentage of volume required to be considered *monofloral bee pollen* (3.4)

3.6
dried bee pollen

bee pollen (3.3) obtained through dehydration to decrease water content under controlled conditions and a temperature no higher than 40 °C without ultraviolet (UV) light exposure

Note 1 to entry: Field-dried bee pollen shall be obtained without direct sunlight and preliminary treatment under clean hot air not exceeding 40 °C.

Note 2 to entry: Further addition of storage preservatives, e.g. SO_2, is not allowed.

Note 3 to entry: Before the drying process, a freezing period of a minimum of 24 h is recommended to kill any moth eggs or fungi.

3.7
frozen bee pollen

bee pollen (3.3) obtained immediately through a freezing process, at a temperature inferior to −18 °C, without any dried treatment until consumed

3.8
lyophilized bee pollen

bee pollen (3.3) produced from *frozen bee pollen* (3.7) after a process of lyophilization

4 Technical requirements and recommendations

4.1 General

The following general requirements and recommendations apply:

a) The bee pollen should be irregular orbicular sphere-like.

b) The bee pollen shall have organoleptic characteristics such as colour, appearance, odour and taste that vary according to the botanical origin.

c) The bee pollen shall be clean without contaminants from the beehive to the final product (live or dead insects, larvae, eggs, etc.) and environmental matter (soil, sand, etc.) and shall be free from any additives. Preserve the bee pollen from the development of other insects and parasites.

4.2 Chemical requirements

The chemical requirements of the product shall meet the criteria specified in Table 1. Regarding chemical composition, maximum values are established only for moisture content and pH (see Table 1). The other classes of compounds are in accordance with the botanical origin of the bee pollen. All the test methods were identified with their protocols. Each of the methods listed in Table 1 and in the annexes may be adapted or replaced provided that the modifications made can be shown to give similar results of at least equivalent quality to those of the basic method listed in the annexes.

Table 1 — Chemical requirements for bee pollen and test methods for each characteristic

Characteristic	Min. or max.	Requirements			Test method
		Type 1	Type 2	Type 3	
Determination method of pollen taxa at bee pollen	—	Limits only for monofloral bee pollen			Annex A
Moisture content with % vacuum drying oven method, in % mass fraction	min.	2	No upper and lower limits defined	2	Clause B.1
	max.	8		8	
Loss on drying content with drying oven method, in % mass fraction	min.	5		5	Clause B.2
	max.	9		9	
Halogen loss on drying analyser	min.	3		3	Clause B.3
	max.	9		9	
Protein content, in % mass fraction	min.	10	7,5	10	Annex C
Sugar content, in % mass fraction	—	No lower and upper limits were defined			Clause D.1
Total sugar, in % mass fraction	min.	15	12	15	Clause D.2
Lipid, in % mass fraction	min.	1,3	1	1,3	Annex E
Determination of pH	min.	3,3			Annex F
	max.	6,30			
Ash, in % mass fraction, by gravimetry	min.	1	0,7	1	Annex G

Key

Type 1 – Dried bee pollen; Type 2 – Frozen bee pollen; Type 3 – lyophilized bee pollen.

4.3 Safety and health requirements

The limit accepted for toxic substances (pyrrolizidine alkaloids, cannabinoids, grayanotoxins, and others) naturally present in some plants (*Rhododendron* spp., *Cannabis* spp. and others) shall be taken into consideration.

5 Test methods

5.1 Reagents

Use only reagents of recognized analytical grade unless otherwise specified.

5.2 Sample collection

Bee pollen is a heterogeneous product; therefore, at least 0,5 % of the batch (minimum 250 g for batches less than 50 kg and 500 g of sample for batches less than 100 kg) shall be collected. A minimum of five representative points (a random set of points that optimally represents a distribution of the bee pollen container) shall be sampled to consider the diversity of the bee pollen. Pack them in a food-grade hermetic container and store them in a frozen condition.

5.3 Transportation from the hive to the factory or laboratory

Put the sample into the sterile sample bottle, stir sufficiently to mix it evenly and put it aside as the sample to be tested.

The collected bee pollen shall come to the laboratory to be stored in a freezer as soon as possible. Using an icebox (with ice inside) for transportation is recommended.

During the harvest, beekeepers shall be aware of the type of packaging material used to transport the product from the apiary to the processing sector or the laboratory for quality control and thus avoiding:

a) crushing of raw material;

b) contamination with dust;

c) transfer of odours and humidity and high temperatures, which can interfere with the quality of the product to be processed. Use only reagents of recognized analytical grade unless otherwise specified.

5.4 Test methods for chemical requirements

Each sample shall represent the collected bee pollen lot.

Bee pollen is very hydrophilic, and its exposure to humidity in the room shall be limited as much as possible. In this context, airtight containers are recommended until the beginning of the analysis.

Bee pollen collection for laboratory samples shall be made from homogenization containers in sterilized jars.

For a better homogenization of the sample, the bee pollen should be ground into a fine powder and stored according to the bee pollen state (dried, frozen and lyophilized).

The sample can be tested in the following different conditions:

- for dried bee pollen, the samples shall be stored at a temperature no higher than 4 °C in a well-conditioned area for no more than six months;
- for frozen bee pollen, the samples shall be stored at −18 °C for no more than six months;
- for lyophilized bee pollen the samples shall be stored at a temperature no higher than 4 °C in a well-conditioned area for no more than six months.

For all analytical procedures, the homogenization of bee pollen pellets is done by grinding in the mill until the bee pollen pellets become a powder (for up to 2 min).

All the test methods are explained in Annexes A to G.

6 Packing, labelling, marking, storage and transportation

6.1 Packing

The bee pollen shall be packed in closed, clean and dry food-grade containers, protected from UV light and made of a material that does not affect the quality of the bee pollen.

6.2 Labelling of the bee pollen plant taxa

It shall be stated in the labelling that the product is MNBP (monofloral) or MTBP (multifloral) type.

For MNBP monofloral bee pollen, only “monofloral bee pollen” with the name of the dominant plant shall appear on the face label (local and Latin name).

For MTBP multifloral bee pollen, the name “multifloral bee pollen” shall appear on the face label. It is optional to include a list of plants on the label (Latin names) for customers to refer to when purchasing. Nevertheless, the complete list is not required and can be limited to a reduced list of different botanical origins.

6.3 Marking

The packages of bee pollen information shall be labelled.

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

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

b) the name and address of the producer and packer, and country or countries of origin;

c) the type of treatment information needed to obtain a dried, field-dried, frozen and lyophilized product;

d) the labelling of the plant taxa in 6.2 (for MNBP is mandatory and for MTBP is optional);

e) the net mass;

f) the harvesting years and the "best-before" date;

g) the batch number and the traceable information of the product; ISO 22005 shall be followed to declare the traceability of bee pollen;

h) nutritional information.

6.4 Storage and transportation

Medium- and long-term storage should be in a shady and cool place or, preferably, within a temperature range of between +2 °C and +5 °C for dried bee pollen or lyophilized bee pollen. Frozen bee pollen shall be stored at less than −18 °C. The maximum shelf life of bee pollen as human food is recommended to be less than 24 months for frozen and lyophilized bee pollen and less than 18 months for dried bee pollen.

Bee pollen produced in different areas and times should be stored separately and given different batch numbers (in a bottle or a box).

Bee pollen shall be transported by cold-chain conditions, e.g. freezing transport (−22 °C to −18 °C) for frozen bee pollen, and transport at a temperature of no more than 25 °C (0 °C to 10 °C is recommended) for dried and lyophilized bee pollen. Transport shall be in sealed containers to avoid moisture pick-up.

Bee pollen shall not be stored and transported with toxic, corrosive materials or materials with a peculiar odour or that can cause contamination.

Annex A
(normative)

Determination method of pollen taxa in bee pollen

A.1 Determination method of pollen taxa in bee pollen (alternative to Clause A.2)

A.1.1 Principle

The method is for the botanical origin identification and qualitative analysis (relative frequencies) of pollen taxa in bee pollen samples.

A.1.2 Consumables

A.1.2.1 Microscopic slide.

A.1.2.2 Lamella.

A.1.2.3 Dissection needle.

A.1.2.4 Centrifuge tube (15 ml and 50 ml).

A.1.3 Reagents

A.1.3.1 Ethanol (70 %) or **H_2SO_4** (5 ‰) (optional).

A.1.3.2 Distilled water.

A.1.3.3 Gelatin (powder) or **Kaiser's glycerol gelatine.**

A.1.3.4 Glycerine or **Kaiser's glycerol gelatine.**

A.1.3.5 Basic fuchsine (powder) (optional).

A.1.4 Apparatus

A.1.4.1 Hot plate.

A.1.4.2 Vortex.

A.1.4.3 Centrifuge.

A.1.4.4 Light microscope, ×400, ×1 000 magnification with oil lens.

A.1.5 Procedure

A.1.5.1 Preparation of basic fuchsine dye (recommended)

Use the following procedure:

a) Put 1 g powder basic fuchsine in a 100 ml beaker.

b) Add 100 ml ethanol (A.1.3.1) and mix completely.

A.1.5.2 Preparation of glycerine gelatine matrix

Use the following procedure of option 1 or option 2:

a) option 1:

  1) Put 7 g of powder gelatine in a 100 ml beaker.

  2) Add 50 ml distilled water.

  3) It is recommended to melt the gelatine at about 50 °C on the hot plate.

  4) After the gelatine melts, add 50 ml glycerine and mix.

  5) Add a few drops of basic fuchsine to the gelatine to dye the glycerol gelatine (which should be dark pink) and mix.

  6) After cooling down, store it in a refrigerator at +4 °C in a capped jar.

b) option 2:

  1) Melt the Kaiser's glycerol gelatine at about 60 °C in the glass bottle.

  2) Add a few drops of basic fuchsin to the gelatine to dye the glycerol gelatine (which should be dark pink) and mix.

  3) After cooling down, store it in a refrigerator at +4 °C in a capped jar.

A.1.5.3 Preparation of bee pollen slides

Use the following procedure:

a) Weigh 5 g ground bee pollen sample into a 50 ml centrifuge tube and add distilled water up to a 30 ml volume.

b) Keep it at room temperature for 20 min.

c) Adjust the volume to 30 ml with distilled water if necessary.

d) Shake and vortex until the pollen suspension is entirely homogeneous.

e) Immediately after the vortex, transfer 400 µl of this suspension into a new 15 ml tube.

f) 1 ml ethanol or 1 ml H_2SO_4 5 ‰ is added and vortexed.

g) The mixture is kept at room temperature for 10 min to 20 min.

h) The suspension is centrifuged for 10 min at least 1 000*g* force (3 500 r/min to 4 000 r/min).

i) Supernatant harvesting: washing with 1 ml distilled water to remove H_2SO_4 5 ‰; if ethanol has been used, this step is not mandatory.

j) Fill distilled water up to 1 ml and vortex well.

k) Immediately after the vortex, a volume necessary for drawing an equal area to the lamella used is pipetted from the mixture (suspension) (e.g. 17 µl to 20 µl is pipetted if a 20 mm × 20 mm or a 24 mm × 24 mm lamella is used, respectively), heated to 40 °C and left until completely dry. The slide in this form, heated to 40 °C, is added to the glycerine:gelatine:fuchsine/Kaiser’s glycerol gelatine:fuchsine to melt (the fuchsine is optional); and then is covered with a lamella and left upside down for 1 h before the microscopic analysis. Then, it is examined with a light microscope.

Steps f) to i) are optional.

A.1.5.4 Evaluation of bee pollen slides

Use the following procedure:

a) To identify pollen grains, type, shape and size of the pollen grain, thickness and ornamentation type of exine, aperture number, place of the apertures on pollen grain, size and type of aperture as well as pore and cracks properties are examined.

b) The identification and counting of pollen are done using an optical microscope under ×1 000 or ×400 amplifications of at least 600 pollen grains, sweeping the entire surface of the lamella into at least six lines from top to bottom. On the lamella, count approximately 100 pollens for each line.

c) If a dominant monofloral bee pollen (MNBP) is suspected, the size of the pollen shall be defined. Without scientific reference, the radius shall be evaluated (average of the polar and equatorial axis).

d) The radius shall be calculated based on the average of 10 pollens. The pollen size shall be defined for all the pollen of less than 20 µm present at least 5 % and the other pollen of more than 20 µm present at least 1 %.

e) For multifloral bee pollen (MTBP): the list of pollens is recommended to be given according to the pollen spectrum.

A.1.6 Calculation

Count at least 600 identified pollens in the visual fields. The percentage of the volume of pollen type or species candidate to be monofloral, V, shall be calculated by using Formula (A.1):

$$V = \frac{X_1 \times r_1^3}{\sum_i^n \left(X_i \times r_i^3\right)} \times 100 \qquad \text{(A.1)}$$

where

X_1 is the relative frequency of pollen type or species candidate to be monofloral;

r_1 is the mean radius of pollen type or species candidate to be monofloral;

X_i is the relative frequency of each pollen type or species present in bee pollen;

r_i is the mean radius of each pollen type or species present in bee pollen;

To be monofloral $V \geq 80$ %.

A.1.7 Precision

The relative deviation of two parallel tests shall be no more than 20 %. Only pollen present in percentages of at least 5% is considered during the calculation of relative deviation.

A.2 Determination method of pollen taxa in bee pollen by acetolysis (alternative to Clause A.1)

A.2.1 Principle

The method is for the botanical origin identification and quantitative analysis of pollen taxa in bee pollen samples.

A.2.2 Consumables

A.2.2.1 Microscopic slide.

A.2.2.2 Lamella.

A.2.2.3 Centrifuge tubes (5 ml and 50 ml).

A.2.2.4 Beakers.

A.2.2.5 Glass rod.

A.2.3 Reagents

A.2.3.1 Distilled water.

A.2.3.2 Gelatin (powder).

A.2.3.3 Glycerine (Kaiser's glycerol gelatine).

A.2.3.4 Glacial acetic acid, analytical grade.

A.2.3.5 Sulfuric acid, 96 %.

A.2.3.6 Acetic anhydride, pure.

A.2.3.7 Glycerol 87 %.

A.2.3.8 Histolac glue or nail lacquer.

A.2.4 Apparatus

A.2.4.1 Hot plate.

A.2.4.2 Vortex.

A.2.4.3 Centrifuge (at least 1 000*g*).

A.2.4.4 Light microscope ×400, ×1 000 magnification with oil lens.

A.2.4.5 Balance.

A.2.4.6 Water bath.

A.2.5 Procedure

A.2.5.1 Preparation of glycerine gelatine matrix (optional)

Use the following procedure:

a) 7 g of powder gelatine is put in a 100 ml beaker.

b) It is completed to 50 ml with distilled water.

c) It is recommended to melt the gelatine at 50 °C on the hot plate.

d) After the gelatine melts, 50 ml of glycerine is added and mixed.

e) After it cools down, it is stored in a refrigerator at +4 °C in a capped jar.

A.2.5.2 Preparation of acetolysis solution

In a beaker, add nine parts of acetic anhydride to one part of concentrated sulfuric acid, e.g. to make 50 ml solution, slowly add 5 ml of sulfuric acid to 45 ml of acetic anhydride and stir well with a glass rod.

A.2.5.3 Preparation of glycerol solution (50 %)

In a beaker, add an equal volume of water and glycerol and stir well with a glass rod.

A.2.5.4 Preparation of bee pollen slides with acetolysis

Use the following procedure:

a) Weigh 5 g ground bee pollen sample into a 50 ml centrifuge tube and add distilled water up to 30 ml volume.

b) Keep it at room temperature for 20 min.

c) Adjust the volume to 30 ml with distilled water if necessary.

d) Shake and vortex until the pollen suspension is entirely homogeneous.

e) Immediately after the vortex, transfer 400 µl of this suspension into a new 5 ml tube.

f) Add 2 ml of glacial acetic acid and vortex.

g) Centrifuge at least 1 000*g* for 10 min and decant.

h) Add 2 ml of the acetolysis solution and vortex well until the pollen disaggregates.

i) Place the tubes in a boiling water bath for 3 min with the lids open or on water bath at 80 °C for 15 min.

j) Add 2 ml of acetic acid for stopping the acetolytic reaction (optional).

k) Centrifuge at least 1 000*g* for 10 min and decant.

l) Add 2 ml of water and shake well with vortex until the pollen disaggregates. Add another 2 ml of water and shake.

m) Centrifuge at least 1 000*g* for 10 min and decant.

n) Add 1 ml of glycerol solution (50 %) and vortex well until the pollen disaggregates.

o) Immediately after the vortex, a volume necessary for drawing an equal area to the lamella used is pipetted from the mixture (suspension) (e.g. 17 µl to 20 µl is pipetted if a 20 mm × 20 mm or a 24 mm × 24 mm lamella is used, respectively), heated to 40 °C and left until completely dry. Keep the slide at 40 °C, add the glycerine:gelatine/Kaiser's glycerol gelatine to melt; and then cover it with a lamella and leave upside down for 1 h before the light microscopic analysis. Seal with histolac glue or lacquer nail. Then, examine the slide with a light microscope.

A.2.5.5 Evaluation of bee pollen slides

Use the following procedure:

a) To identify pollen grains, type, shape and size of the pollen grain, thickness and ornamentation type of exine, aperture number, place of the apertures on pollen grain, size and type of aperture as well as pore and cracks properties are examined.

b) The identification and counting of pollen are done using an optic microscope under ×1 000 or ×400 amplifications of 600 pollen grains, sweeping the entire surface of the lamella into six lines from top to bottom. On the slide, count approximately 100 pollens for each row.

c) If a dominant bee pollen (MNBP) is suspected, the size of the pollen shall be defined. Without scientific reference, the diameter shall be evaluated (average of the polar and equatorial axis).

d) The diameter shall be calculated based on the average of 10 pollens. The pollen size shall be defined for all the pollen of less than 40 µm present at least 5 % and the other pollen of more than 40 µm present at least 1 %.

e) For multifloral bee pollen (MTBP): the list of pollens is recommended to be given according to the pollen spectrum.

A.2.6 Calculation

Count at least 600 identified pollens in the visual fields. The percentage of the volume of pollen type or species candidate to be monofloral, V, shall be calculated by using Formula (A.2):

$$V = \frac{X_1 \times d_1^3}{\sum_i^n \left(X_i \times d_i^3 \right)} \times 100 \qquad \text{(A.2)}$$

where

X_1 is the relative frequency of pollen type or species candidate to be monofloral;

d_1 is the mean diameter of pollen type or species candidate to be monofloral;

X_i is the relative frequency of each pollen type or species present in bee pollen;

d_i is the mean diameter of each pollen type or species present in bee pollen;

To be monofloral $V \geq 80$ %.

NOTE The use of diameter instead of radius will not change the result of the formula.

A.2.7 Precision

The relative deviation of two parallel tests shall be no more than 20 %. Only pollen present in percentages of at least 5% is considered during the calculation of relative deviation.

Annex B
(normative)

Determination of moisture content

B.1 Vacuum drying oven method (preferred)

B.1.1 Principle

The method determines the moisture content of bee pollen under a vacuum drier with hot circulating air. The moisture content is determined by a differential weighing before and after drying.

B.1.2 Consumables

B.1.2.1 Weighing dish, height 25 mm to 30 mm, diameter 35 mm to 50 mm.

B.1.3 Apparatus

B.1.3.1 Vacuum drying oven.

B.1.3.2 Analytical balance, capable of weighing to the nearest 0,000 1 g.

B.1.4 Procedure

Use the following procedure:

a) Weigh accurately 2 g (precision to 0,000 1 g) of the bee pollen sample.

b) Put into a weighing dish, dry to constant mass, spread evenly and put it in a vacuum drying oven.

c) Vacuum the drying oven (with the approximate pressure between 0,04 MPa and 0,053 MPa), and dry at 60 °C ± 5 °C for 4 h.

d) Take out the weighing dish and put it in the drying oven or desiccator and weigh after cooling for 30 min.

e) Repeat the drying process for another 2 h, cool for 30 min and weigh.

f) Repeat the procedure until the mass difference between two consecutive measurements is no more than 2 mg, namely, until a constant mass is achieved.

B.1.5 Calculation

The moisture content in bee pollen, X_1, given by loss on drying content, in % mass fraction, is calculated by using Formula (B.1):

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

where

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

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

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

B.1.6 Precision

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

B.2 Loss on drying with oven method

B.2.1 Principle

The method determines the loss on drying content of bee pollen under a drier oven with hot circulating air. The loss on drying content is determined by a differential weighing before and after drying.

B.2.2 Consumables

B.2.2.1 Weighing dish, height 25 mm to 30 mm, diameter 35 mm to 50 mm.

B.2.3 Apparatus

B.2.3.1 Drying oven.

B.2.3.2 Analytical balance, capable of weighing to the nearest 0,000 1 g.

B.2.4 Procedure

Use the following procedure:

a) Weigh accurately 2 g (precision to 0,000 1 g) of the bee pollen sample, put in a weighing dish having dried to a constant mass, and spread evenly.

b) Put in the drying oven for at least 4 h, and dry at 105 °C ± 5 °C until a constant mass is achieved precisely, similar to the method in Clause B.1.

B.2.5 Calculation

The moisture content in bee pollen, X_1, given by loss on drying content, in % mass fraction, is calculated by using Formula (B.2):

$$X_1 = \frac{m_1 - m_2}{m_1 - m_3} \times 100 \qquad \text{(B.2)}$$

where

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

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

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

B.2.6 Precision

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

B.3 Halogen drying

B.3.1 Principle

The method determines the loss on drying content of pollen with halogen drying. This method is a weighing-drying method in which the samples are dried until a constant mass is achieved using the halogen radiator principle.

B.3.2 Consumables

B.3.2.1 Weighing dish, height 20 mm to 30 mm, diameter approximately 90 mm (the height and diameter of the weighing dish should be suitable for the halogen heated moisture analyser use.

B.3.3 Apparatus

B.3.3.1 Moisture analysers with halogen heating.

B.3.4 Procedure

B.3.4.1 Put the weighing dish in the moisture analyser and run the programme (B.3.4.2) while the weighing dish is empty. Tare the weighing dish on the moisture analyser and weigh accurately 2 g (precision to 0,001 g) of the bee pollen sample. Spread the homogenized pollen evenly over the surface of the weighing dish. Run the programme (B.3.4.2).

B.3.4.2 Programme:

- temperature: 105 °C;
- time for constant mass decision.

B.3.4.3 Register the result displayed by the moisture analysers directly after the analysis is finished.

B.3.5 Precision

The relative deviation of parallel experiments shall not be more than 1 %.

Annex C
(normative)

Determination of protein in bee pollen

C.1 Principle

The method determines the total protein content in bee pollen samples.

C.2 Consumables

C.2.1 Digestion tubes: 250 ml.

C.2.2 Titration flask: 500 ml graduated Erlenmeyer flask (for collection and titration of distillate).

C.2.3 Weighing paper: low N.

C.3 Reagents and materials

C.3.1 Concentrated sulfuric acid: (approximately 95 % to 98 %) reagent grade.

C.3.2 Mixed reagent of copper sulfate and potassium sulfate: weigh 0,4 g copper sulfate and 7 g potassium sulfate, put it in the mortar, mix evenly, and grind finely to use as Kjeldahl tablets free of mercury and selenium 3,5 g.

C.3.3 Mixed indicator: dissolve 100 mg methyl red in 100 ml methanol and 100 mg bromocresol green in 100 ml methanol.

C.3.4 Boric acid solution, 4 % (mass/volume): dissolve 40 g boric acid in 0,5 l to 0,6 l hot deionized water. Mix and add more hot deionized water to a volume of 1 l.

C.3.5 Dilute hydrochloric acid: measure 5,7 ml concentrated hydrochloric acid and dilute to 100 ml with distilled water.

C.3.6 Hydrochloric acid standard solution (0,1 mol/l): dilute 10 times before using.

C.4 Apparatus

C.4.1 Analytical balance, capable of weighing to the nearest 0,000 1 g.

C.4.2 Digestion block: Aluminium alloy block with adjustable temperature device for measuring and controlling block temperature.

C.4.3 Distillation units: with automatic addition of NaOH and H_2O, to accept 250 ml digestion tubes and 500 ml titration flask.

C.5 Digestion

Use the following procedure:

a) Weigh approximately 1 g of bee pollen sample onto a tared, low N weighing paper. Fold the paper around the material and drop it into a Kjeldahl tube.

b) Add 20 ml sulphuric acid 95 % to 98 % + 2 Kjeldahl tablets.

c) Attach heat side shields to tube rack.

d) Place the fume manifold tightly on the tubes and turn the water aspirator on completely.

e) Set the temperature setting to 40 % and place the tube rack in the block.

f) After 15 min, the temperature setting is brought to 70 %.

g) After 15 min, the temperature setting is brought to 100 %.

h) After about 30 min to 45 min, the heating continues until the samples turn clear green. The burning process continues for 30 min after the colour change.

i) Then turn off the device and let the tubes cool for 15 min.

j) After 10 min, turn the water aspirator down until acid fumes are just contained within the exhaust hood.

k) A condensation zone should be maintained within the tubes. After the bulk of the sulfur oxide’s fumes are produced during the initial stages of digestion, reduce vacuum source to prevent loss of sulphuric acid.

l) Digest an additional 50 min. The total digestion time is approximately 60 min.

m) Let the tubes cool. Add distilled water to each tube to a total volume of approximately 80 ml.

C.6 Distillation

Use the following procedure:

a) Place 40 % NaOH in the alkali tank of the distillation unit.

b) Adjust the volume dispensed to 50 ml.

c) Attach a digestion tube containing diluted digest to the distillation unit.

d) Place a graduated 500 ml Erlenmeyer titration flask containing 25 ml H_3BO_3 solution, two drops of methyl red solution and three drops of bromocresol green.

e) Steam distil until ≥ 150 ml distillate is collected (5 min) and remove the receiving flask.

f) Alternatively, the distillation is carried out with a distillation unit. The volumes that can be used are 50 ml H_2O, 90 ml NaOH, and 60 ml H_3BO_3, but they can be changed according to the distillation unit used.

C.7 Titration

Titrate H_3BO_3 receiving solution with standard 0,1 mol/l HCl to the violet end point. Record millilitres of HCl to at least the nearest 0,05 ml or perform titration using an automatic titrator, using 0,25 M sulfuric acid for protein-rich matrices, up to a pH of 4,65.

C.8 Calculation

The protein content in bee pollen, N, in % mass fraction, is calculated by using Formula (C.1):

$$N = \frac{(V_s - V_b) \times M \times 14{,}01}{m \times 1\,000} \times 5{,}6 \times 100 \quad \text{(C.1)}$$

where

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

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

M is the concentration of the standard acid solution, in mol/l;

14,01 is the atomic mass of N;

m is the mass of the sample, in g;

1 000 is the factor to convert mg/g to %;

5,6 is the factor to convert N to proteins.

C.9 Precision

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

Annex D
(normative)

Determination of sugars content in bee pollen

D.1 High performance liquid chromatography with refractive index detector HPLC-RID method (preferred method)[1]

D.1.1 Principle

The method is the quantitative analysis of fructose, glucose, sucrose, maltose, isomaltose, maltotriose, erlose and melezitose saccharides at the HPLC-RID system.

D.1.2 Consumables

D.1.2.1 Pipet tips.

D.1.2.2 Glass vials and caps.

D.1.2.3 Sample preparation tubes (centrifuge tubes/15 ml falcons).

D.1.2.4 Polyvinylidene fluoride (PVDF) membrane filter, pore size 0,22 µm.

D.1.2.5 2 ml disposable syringes.

D.1.3 Reagents

D.1.3.1 Ultrapure water.

D.1.3.2 Methanol, HPLC purity or analytical purity > 99,0 %.

D.1.3.3 Acetonitrile, HPLC purity or analytical purity > 99,0 %.

D.1.3.4 Hexane, HPLC purity or analytical purity > 99,0 %.

D.1.3.5 Analytical standards; fructose, glucose, sucrose, maltose, isomaltose, maltotriose, erlose, melezitose, HPLC purity or analytical purity > 98,0 %.

D.1.3.6 Carrez solution I: 15 g of potassium hexacyanoferrate (II) trihydrate, $K_4Fe(CN)_6{\cdot}3H_2O$ dissolved in 100 ml of deionized water.

D.1.3.7 Carrez solution II: 30 g of zinc acetate dihydrate, $Zn(CH_3COO)_2{\cdot}2H_2O$ dissolved in 100 ml of deionized water.

D.1.3.8 Mobile phase: acetonitrile: water (83:17 volume/volume).

D.1.3.9 LC strong wash: acetonitrile: water (1:99 volume/volume).

D.1.3.10 LC weak wash: acetonitrile.

D.1.4 Apparatus

D.1.4.1 HPLC operated by computer software and equipped with: pump, degasser, autosampler, column oven, and refractive index detector (RID).

D.1.4.2 Chromatographic column, with amino-modified phase (100 Å, 5 µm particle size, L: 100 mm and internal diameter 3 mm).

D.1.4.3 Analytical balance, capable of weighing to the nearest 0,000 1 g.

D.1.4.4 Mechanical shaker.

D.1.4.5 Automatic dispenser.

D.1.4.6 Laboratory glassware (volumetric flasks).

D.1.4.7 Degasser.

D.1.5 Storage and stability conditions

Use the following procedure:

a) Diluted standard mixtures for calibration can be stored at 4 °C for two days.

b) The stock standard mixture can be stored at −18 °C for six months.

c) Carrez solution shall be kept at room temperature for a maximum of three months.

D.1.6 Sample treatment

Use the following procedure:

a) Weigh accurately 1 g ± 0,00 1 g of bee pollen in a 15 ml falcon tube.

b) Add 6,3 ml water and 2,1 ml of acetonitrile.

c) Vortex is applied for getting the homogenized bee pollen-water-acetonitrile suspension.

d) Add 0,2 ml Carrez 1 and 0,2 ml Carrez 2 reagents, and mix the sample immediately upon the Carrez addition.

e) To get rid of the protein pellet, centrifuge for 10 min at 3 000*g*.

f) Transfer 5 ml of the resulting supernatant to a new 15 ml falcon tube and defat with 5 ml hexane addition.

g) Vortex the sample for 1 min after hexane addition and centrifuge for 10 min at 3 000*g*.

h) Remove the hexane layer and repeat this step twice more.

i) Filter the final defatted, sugar-included lower layer using 0,22 µm PVDF syringe filter to a glass vial and inject into the LC system.

j) Samples shall be injected on the same day and kept at 4 °C until analysis for a maximum of 24 h.

D.1.7 Chromatography conditions

Use the following procedure:

a) Flow rate: 1,0 ml/min.

b) Column and detector temperature: 35 °C.

c) Autosampler temperature: 4 °C.

d) Injection volume: 10 µl.

e) Run time: 10 min (can be optimized).

D.1.8 Calibration

Purity levels shall be taken into account before weighing.

Sugar Solution 1 (S1) (stored at +4 °C for two days): In a 100 ml beaker, 0,11 g ± 0,05 g of fructose, 0,11 g ± 0,05 g of glucose, 30 mg ± 0,05 mg of sucrose, 30 mg ± 0,05 mg of maltose, 30 mg ± 0,05 mg of maltotriose and 30 mg ± 0,05 mg of melezitose are weighed. Dissolve the content in ultrapure water and add 2,5 ml acetonitrile. In a 10 ml volumetric flask, complete to 10 ml with ultrapure water.

Sugar solution 2 (S2) (stored at +4 °C, prepared before use): Weigh 10 mg ± 0,05 mg of isomaltose and erlose. Dissolve the mixture in 750 µl ultrapure water and transfer into a 1 ml vial. Add 250 µl acetonitrile.

Mix 900 µl of S1 and 100 µl of S2 in a 1 ml vial and vortex. Filter this solution using a 0,22 µm PVDF filter before injection.

Based on the preparation above, the final concentration of each sugar in the vial is as follows:

- fructose and glucose: 9,9 mg/ml;
- sucrose, maltose, maltotriose and melezitose: 2,7 mg/ml;
- isomaltose and erlose: 1,0 mg/ml.

At least five dilutions are needed for the calibration curve.

D.1.9 Calculation

The concentration of the sugar i in the sample, C_i, in mg/ml, is calculated by using Formula (D.1):

$$C_i = K_i \times A_i \tag{D.1}$$

where

K_i is the response factor of sugar i, which is calculated from the slope of calibration curve constructed by the area against the concentration of the standard solutions;

A_i is the area of sugar i in the sample.

The total sugar in bee pollen, T_i, in %, is calculated by using Formula (D.2):

$$T_i = C_i \times 8{,}8/m \times 100 \tag{D.2}$$

where

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

m is the mass of the sample, in mg (1 000).

D.1.10 Precision

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

The correlation coefficient of the linear dependence of sugar concentration and peak area in the working range reached the value of over 0,995 for all the analysed sugars.

D.2 Titration method

D.2.1 Principle

The method is the quantitative analysis of total sugar content in bee pollen, using titration methodology.

D.2.2 Consumables

D.2.2.1 150 ml conical bottles.

D.2.2.2 100 ml, 250 ml and 1 000 ml volumetric flasks.

D.2.2.3 25 ml acid burette

D.2.3 Reagents

D.2.3.1 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.2.3.2 Potassium ferrocyanide, ρ = 106 g/l. Weigh 10,6 g hydrochloric acid, add distilled water and dilute to 100 ml.

D.2.3.3 Hydrochloric acid, c = 6 mol/l. Weigh 50 ml hydrochloric acid (w = approximately 36 % to 38 %), add distilled water and dilute to 100 ml.

D.2.3.4 Methyl red indicator, ρ = 1 g/l, ethanol solution.

D.2.3.5 Sodium hydroxide solution, ρ = 200 g/l. Weigh 20 g hydrochloric acid, add distilled water and dilute to 100 ml.

D.2.3.6 Alkaline cupric tartrate titration solution (TS) solution A. Dissolve 15 g copper sulfate ($CuSO_4 \cdot 5H_2O$) and 0,05 g methylene blue in 1 000 ml water.

D.2.3.7 Alkaline cupric tartrate titration solution (TS) solution B. Weigh 50 g potassium sodium tartrate and 75 g sodium hydroxide, dissolve with distilled water, add 4 g potassium ferrocyanide and dilute to 1 000 ml with distilled water.

D.2.3.8 Glucose standard solution, ρ = 1 g/l. Weigh accurately 1 g pure glucose (specific rotation is approximately +52,5° to +53°) with constant mass after it is dried at a temperature from 98 °C to 100 °C, dissolve with distilled water, add 5 ml hydrochloric acid (c = 6 mol/l) and dilute to 1 000 ml with distilled water.

D.2.4 Apparatus

D.2.4.1 Electric-heated thermostatic water bath, with temperature fluctuation of ±1 °C.

D.2.4.2 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.2.5 Procedure

D.2.5.1 Sample treatment

Use the following procedure:

a) Weigh 3 g (accurate to 0,001 g) of bee pollen sample into a 250 ml volumetric flask, add 50 ml of distilled water, vortex fully to generate homogenized bee pollen-water.

b) Slowly add 5 ml of zinc acetate solution (D.2.3.1) and 5 ml of potassium ferrocyanide solution (D.2.3.2), dilute to the mark with distilled water, mix well and let stand for 30 min.

c) Filtrate with dry filter paper, discard the first filtrate and take the subsequent filtrate for use.

D.2.5.2 Hydrochloric acid hydrolysis

Use the following procedure:

a) Measure 50 ml treated sample into 5 ml of hydrochloric acid (D.2.3.3), heated in a water bath at 68 °C to 70 °C for 15 min.

b) Add two drops of methyl red indicator solution (D.2.3.4). After cooling, neutralize fully to neutral with 200 g/l sodium hydroxide solution (D.2.3.5).

D.2.5.3 Calibration of alkaline cupric tartrate TS solution

Use the following procedure:

a) Weigh accurately 5 ml each of alkaline cupric tartrate TS solutions A (D.2.3.6) and B (D.2.3.7).

b) Put them in 150 ml conical bottles, add 10 ml distilled water, add approximately 9 ml glucose standard solution (D.2.3.8) from a burette, heat to the boiling point within 2 min and keep adding standard glucose solution at the speed of 1 drop per 2 s when it is boiling.

c) 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 each from solutions A and B) of alkaline cupric tartrate TS solution.

D.2.5.4 Titration

Use the following procedure:

a) Pipette 5,0 ml each of alkaline cupric tartrate TS solutions A (D.2.3.6) and B (D.2.3.7). Put them in a 150 ml conical bottle, add 10 ml of distilled water, control the heat to a boiling point within 2 min and keep boiling.

b) Add the sample solution from the burette at a fast and slow speed, and keep it boiling.

c) When the colour of the solution becomes lighter, titrate at a speed of 1 drop per 2 s until the blue colour of the solution fades as the end point.

d) Record the consumed volume of the sample solution.

D.2.6 Calculation

The total sugar content in bee pollen (counted by glucose), X_2, in % mass fraction, is calculated by using Formula (D.3):

$$X_2 = \frac{m_1}{m \times V / 250 \times 1\,000} \times 100 \qquad \text{(D.3)}$$

where

m_1	is the mass of alkaline cupric tartrate TS, the mass of which 10 ml alkaline cupric tartrate TS (5 ml each from solutions A and B) equals to glucose, in ml;
m	is the mass of the sample, in g;
V	is the volume of sample solution consumed in titration, in ml;
250	is the constant volume, in ml.

D.2.7 Precision

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

Annex E
(normative)

Determination method of lipids

E.1 With Soxhlet device (reference method)

E.1.1 Principle

The method determines the total lipids content using a Soxhlet device.

E.1.2 Consumables

E.1.2.1 Boiling stones.

E.1.2.2 Cellulose cartridge.

E.1.2.3 Round bottom glass flasks.

E.1.2.4 Measuring cylinders.

E.1.3 Reagents

E.1.3.1 Diethyl ether, distilled in glass, with a boiling point of 30 °C to 60 °C.

E.1.4 Apparatus

E.1.4.1 Soxhlet device.

E.1.4.2 Rotary evaporator.

E.1.4.3 Desiccator.

E.1.4.4 Drying oven.

E.1.5 Procedure

Use the following procedure:

a) Place boiling stones in an extraction bottle and keep them in the oven at 105 °C for 1 h.

b) Then, place the extraction bottle in a desiccator and allow to cool for 30 min.

c) After cooling, weigh the extraction bottle and note the value (m_1).

d) Weigh approximately 3,0 g of bee pollen into the paper cartridge (m_3).

e) Place the cartridge in the Soxhlet extractor.

f) Add 160 ml diethyl ether into the glass flasks (brought to a constant mass) and connect to the Soxhlet extractor.

g) All water connections of the extractor are checked, and water reaches the system. The heating system is turned on, and lipid extraction is performed at 40 °C for 7 h.

h) At the end of the period, the extraction flask with lipids is placed in the rotary evaporator, and all solvent is removed.

i) The flask is placed in the oven at 105 °C until it is brought to a constant mass. It is taken into the desiccator, allowed to cool down and weighed (m_2).

j) Per cent lipid amount is calculated.

E.1.6 Calculation

The total lipid in bee pollen, X_3, , in % mass fraction, is calculated by using Formula (E.1):

$$X_3 = \frac{m_2 - m_1}{m_3} \times 100 \tag{E.1}$$

where

m_1 is the mass of the extraction bottle which is dried until a constant mass is achieved, in g;

m_2 is the mass of the extraction bottle after extraction and drying, in g;

m_3 is the bee pollen portion mass, in g.

E.1.7 Precision

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

NOTE This method can be replaced by the automatic method (see Clause E.2).

E.2 With automatic device

E.2.1 Principle

The method determines the total lipids content using an automatic method.

E.2.2 Consumables

E.2.2.1 Boiling stones.

E.2.2.2 Extraction bottle (round or flat bottom).

E.2.2.3 Cellulose cartridge.

E.2.2.4 Measuring cylinders.

E.2.3 Reagents

E.2.3.1 Diethyl ether, distilled in glass, with a boiling point of 30 °C to 60 °C.

E.2.4 Apparatus

E.2.4.1 Automatic device.

E.2.4.2 Rotary evaporator.

E.2.4.3 Drying oven.

E.2.5 Procedure

Use the following procedure:

a) Place boiling stones in an extraction bottle and keep them in the oven at 105 °C for 1 h.

b) Then, place the extraction bottle in a desiccator and keep cooling for 30 min.

c) After cooling, weigh the extraction bottle, and note the value (m_1).

d) Weigh approximately 2,5 g bee pollen (exactly note the weighed amount) and put into the cellulose cartridge (m_3).

e) Place the cartridge in the extractor.

f) Pour a volume of solvent into the solvent reservoir of the extraction apparatus until the sample can be in contact with this solvent.

g) Check all water connections of the extractor and confirm water reaches the system. Turn on the heating system, perform the lipid extraction at 110 °C for 45 min (15 min in the boiling position and 30 min in the rinsing position). Extraction temperature settings can differ; consult the manufacturer's operating instructions.

h) At the end of the period, place the extraction bottle in fume cupboards and let remove the last residues of solvent.

i) Keep the extraction bottle in the oven at 105 °C, cool down in the desiccator and repeat until it is brought to constant mass. Register the final mass (m_2).

The period given in step g) is generally sufficient. This duration shall be verified. After removing the extraction bottle, replace it with a new dried one that has been previously weighed, extract for an additional time (30 min or 1 h) and carry out the operations described in steps e) to i). Verify that the increase in mass of the extraction bottle after the end of step i) does not exceed 25 mg. If not, repeat a third extraction.

E.2.6 Calculation

The total lipid content in bee pollen, X_3, in % mass fraction, is calculated by using Formula (E.2):

$$X_3 = \frac{m_2 - m_1}{m_3} \times 100 \qquad \text{(E.2)}$$

where

m_1 is the mass of an empty cup which is dried until the constant mass, in g;

m_2 is the mass of cup plus the fat residue drying, in g;

m_3 is the bee pollen portion mass, in g.