Chemical composition and content of free tryptophan in Slovenian bee pollen

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Summary

Bee pollen is a good source of different nutrients that are important for humans. The composition varies depending on its botanical origin, region and country. The aim was to characterize, for the first time, bee pollen from Slovenia. Thirty-two samples of bee pollen were collected during the 2014 season. Contents of water, protein, fat and ash were determined, and the total carbohydrate content and energy value were calculated. Sixty-five different botanical families or species were identified in these samples. Fresh Slovenian bee pollen contained on average 227.3 $g \cdot kg^{-1}$ of water, 174.6 $g \cdot kg^{-1}$ of proteins, 73.6 $g \cdot kg^{-1}$ of fat, 20.6 $g \cdot kg^{-1}$ of ash, and 503.8 $g \cdot kg^{-1}$ of total carbohydrate, with an average energy value of 14.3 $MJ \cdot kg^{-1}$. Our data were compared with similar studies and with a draft for the international basic composition requirements for bee pollen in human nutrition. Significant differences were obtained between fat content and energy values across the different months of bee pollen collection. The free tryptophan content was determined in eight unifloral bee pollen samples, and was found to range there from 0.028 $g \cdot kg^{-1}$ to 0.197 $g \cdot kg^{-1}$ fresh weight. These data serve as an indication of the nutritional quality of Slovenian bee pollen.

Keywords

bee pollen; chemical composition; nutritional value; free tryptophan; Slovenia

Pollen is a microscopic structure that is characteristic and specific for every single botanical type. Pollen can vary in terms of its morphological characteristics, such as particle size, form, openings and colour, which are also important for the identification of the plant genus or species [1]. Plants produce pollen in four elongated pollen pouches, known as the anthers. Pollen contains gametophytes, which are the plant male reproductive organs that are the basis for sexual reproduction in plants [2]. Plant propagation is carried out in several ways, with plants mostly pollinated by the wind or by insects.

Among insects, bees are extremely important, having an irreplaceable role in the maintenance of biodiversity and pollination of various crops [2]. Bee body is covered with hairs, and when a bee touches the anthers, its body becomes covered with the pollen dust [2]. Bees accumulate pollen and moisturize it with saliva and nectar. The accumulated pollen grains usually contain approximately 10% nectar [3]. With this process, they enrich the pollen with their enzymes and compress it into two pollen baskets on their hind legs, in which way they form two 'pallets' of pollen. Bees bring these pallets into the beehive. Honey represents a source of energy to the bee colony, while pollen represents the main source of other important nutrients for the bees [1, 3–7].

Bee pollen contains high amounts of carbohydrates, essential amino acids, saturated and unsaturated fatty acids, minerals (e.g. Zn, Cu, Fe) and vitamins (e.g. pro-vitamin A, vitamin E, niacin, thiamin, folic acid, biotin). The contents of these ingredients depend on the botanical origin of the pollen [3]. The presence of these components

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shows that bee pollen can also be used for human nutrition [3]. For the collection of bee pollen, beekeepers install pollen traps at the entrance or at the bottom of the bee hive and, in this way, they can collect the bee pollen for human consumption.

Bee pollen has particular antioxidant, anti-inflammatory and antimicrobial activities, and animal studies have shown that it has benefits against anemia, arteriosclerosis, osteoporosis and allergies [3]. Indeed, bee pollen is used in the diet of some people, because of its nutritional value and its functional characteristics [8], and because it is considered to have a good impact on human health. Knowledge about the functional properties and nutritional value of bee pollen and its impact on certain medical conditions has promoted increased attention to this bee product among consumers [9–13].

Bee pollen is a good source of essential amino acids like tryptophan (Trp), which is involved in protein biosynthesis and is also a precursor of serotonin. Sources of Trp are considered very important for a supply of Trp to reduce depression and anxiety [3], particularly as in its free form Trp can be used immediately for the body needs. Free Trp can be detected without hydrolysis, although hydrolysis under acidic conditions is necessary when bound Trp is determined. There is little information from previous studies about total Trp levels in bee pollen [14, 15]. Appropriate methods for sample preparation must thus be used to preserve Trp levels.

Amino acids are mostly located within the outer layer of the pollen, which is known as exine. The exine contains sporopolenin, which is a very stable biopolymer that protects pollen grains from dehydration. The sporopolenin wrapper is thinner in some places. The thinner places serve for germination of plants, and the thinner outer areas can be helpful at the extraction of amino acids from the inner side of the pollen grain. A combination of a suitable solvent and ultrasound treatment facilitates the extraction of amino acids from pollen grains [14, 15].

With bee pollen being used in human nutrition and for therapeutic purposes, its quality criteria need to be established. A project called Apifresh was run from 2010 to 2013, where the aim was to provide European beekeepers with scientific and technological aids to improve the quality of European bee pollen. This project included development of quality standards for European bee pollen, definition of the analytical methods necessary, determination of bee pollen authenticity and its health enhancing components, as well as development of a set of best-practice guidelines for beekeepers, to improve the quality of bee pollen in all phases of the production chain: harvesting, collecting, storage, transportation and presentation on the market [16, 17]. To provide healthy and safe food for the consumer, a set of standards for this bee product is required.

Quality criteria for bee pollen for human consumption at a national level have been established in Brazil [18], Bulgaria [19], Poland [19] and Switzerland [20]. On the basis of studies from different countries, an international proposal for the quality criteria for dry bee pollen used for human nutrition was also put forward by Campos et al. [3]. However, Slovenia has no legislation concerning the quality criteria for bee pollen for human consumption.

As the composition of bee pollen shows great specificity, data from studies carried out in Australia [21], Brazil [1], China [22], Portugal [23], South Africa [7] and Spain [5] cannot be used for direct extrapolation to Slovenia. The data from previous studies on chemical composition of bee pollen showed very different composition according to the botanical origin, region and country [22], and also according to the period of collection [24]. No direct information on the chemical composition of Slovenian bee pollen is available.

Slovenia has been designated as a protected region, whereby only the use of Carniolan honey bees (*Apis mellifera carnica*, Pollmann, 1879) is permitted. Slovenian beekeepers produce their bee pollen mostly through four months of the year, and still in small quantities. As the basic chemical composition of Slovenian bee pollen remains unknown, this creates problems for the motivation of beekeepers, to encourage them to start to collect this bee product, and also to increase the commercial value of this bee product. The present study provides, for the first time, data on the chemical composition of some bee pollens from Slovenia, and as such, it provides an important contribution to the establishment of international bee pollen quality criteria.

MATERIALS AND METHODS

Samples

Thirty-two samples of bee pollen from Carniolan bees (*A. mellifera carnica*) were collected in the beekeeping season in 2014, over four months from April to July. After collection, the samples were frozen at –18 °C. For the analyses, the mixed bee pollens were used, as these are the more directly relevant bee pollens as collected by beekeepers and as used in human nutrition. For the detection

of the free Trp levels in bee pollen, eight unifloral samples from these mixed bee pollen samples were selected based to their colour, and then analysed. Prior to the chemical analyses, the pollen pellets were ground.

Botanical identification

All of the samples were identified regarding their botanical sources. A sample of 2 g (~300 pollen grains) was considered to be representative for the mixed bee pollen samples. If more than 80% of a sample was of a specific botanical origin, the sample was considered to be unifloral [3]. The analyses for the botanical origins were carried out according to VON DER OHE et al. [25].

Chemical composition

The water content determination was carried out gravimetrically, with drying to constant weight, using a laboratory dryer (~6 h at 105 °C), according to AOAC Method No. 925.09 [26].

Protein content was determined by using the Kjeldahl method and a conversion factor of 6.25 was used, according to AOAC Method No. 981.10 [26].

The fat determinations were carried out using extraction in a Soxhlet apparatus with petroleum ether as the solvent, according to AOAC Method No. 963.15 [26].

The ash content determination was carried out gravimetrically, after incineration at 550 °C to constant weight, according to AOAC Method No. 920.181 [26].

The total carbohydrate (*TC*) content in the fresh bee pollen was obtained from the differences between the other parameters, according to Eq. 1:

$$TC = 100 - (m_W + m_P + m_F + m_A) \quad (1)$$

where m_W is the content of water, m_P the content of protein, m_F the content of fat, and m_A the content of ash (expressed in grammes per kilogram).

Energy value

The energy value (E) was determined according to Eq. 2:

$$E = 17(m_P + m_C) + 37(m_F) \quad (2)$$

where m_P is the content of protein, m_C is the content of total carbohydrate, and m_F is the content of fat (expressed in grammes per kilogram).

E (expressed in megajoules per kilogram) was calculated based on the composition, using the energy conversion factors: 17 MJ·kg^{-1} for protein and carbohydrate, and 37 MJ·kg^{-1} for fat, respectively [27].

Sample preparation for determination of free tryptophan

The extraction of the bee pollen samples was done using different solvents: MilliQ water (Milipore, Billerica, Massachusetts, USA), methanol (Sigma Aldrich, St. Louis, Missouri, USA), MilliQ water : methanol (90 : 10, v/v), and sodium phosphate (Merck, Darmstadt, Germany) buffer (pH 6.75). The extraction was carried out at ultrasonificiation during 10 min using an ultrasound bath, with 25.0 mg pollen sample being combined with 1.25 g solvent. For testing the extraction conditions, the unifloral bee pollen from Brassicaceae was used.

Free tryptophan

The Trp content was determined using HPLC (Knauer, Berlin, Germany), with an electrochemical detector Coulochem III (ESA, Chelmsford, Massachusetts, USA) and a coulometric analytical cell 5011 (ESA). A Purospher STAR RP-18 column was used (5 μm × 150 mm × 4.6 mm; Merck). The mobile phase was 50 mmol·l^{-1} phosphate buffer (pH 6.75). For the preparation of the mobile phase, 6.3 g sodium phosphate (Merck) was dissolved in 1000 ml MilliQ water (Millipore) and then a few drops of orthophosphoric acid (Merck) were added to lower the pH to 6.75. The flow rate was 1 ml·min^{-1}, and the injection volume was 10 μl, with the cell potentials of E_1 = 200 mV and E_2 = 600 mV. A commercial standard of L-Try (Sigma Aldrich) at a stock concentration of 122 μg·ml^{-1} was used for construction of the free Trp calibration curve (R^2 = 0.996).

Statistical analysis

The results were expressed as mean ± standard deviation (*SD*). Differences were tested using one-way analysis of variance (ANOVA) followed by Duncan's test, with significance set at α = 0.05. The IBM SPSS Statistics software was used (IBM, Armonk, New York, USA).

RESULTS AND DISCUSSION

Identification of bee pollen plant sources

Sixty-five different botanical families or species were identified in the bee pollen samples, with nine of these being dominant. According to the collection month through the season, these were:

- April: *Fraxinus ornus* (40% of samples), *Salix* spp. (20%), Brassicaceae (20%);
- May: *Fraxinus ornus* (18%), Brassicaceae (9%), Brassicaceae type *Brassica* (18%), Asteraceae type T (9%);

– June: *Castanea sativa* (50%), *Tilia* spp. (10%), Asteraceae type T (10%), *Plantago* spp. (10%);
– July: *Plantago* spp. (14%), *Trifolium repens* (29%).

The majority of the remaining different botanical families and species thus represented the less prevalent, although potentially still important, minor pollens, at generally < 15%. The botanical identification of bee pollens collected in different months is shown in Fig. 1–4.

Chemical composition of bee pollens

Results of the chemical analyses of bee pollen samples are shown in Tab. 1. The data are expressed on the basis of fresh weight (FW) and dry weight (DW), as indicated. The water content of the fresh bee pollen was from 157.0 g·kg^{-1} to 292.0 g·kg^{-1}. Indeed, fresh bee pollen is known to be very hygroscopic and to contain from 200.0 g·kg^{-1} to 300.0 g·kg^{-1} water [3, 28]. Therefore, the environmental humidity can lead to increased water content in bee pollen if it is not collected daily [23]. To achieve stability for bee pollen, beekeepers dry it, which should be done under controlled conditions, with a temperature not higher than 30–35 °C. Hot air circulation at a temperature that does not exceed 40–45 °C is recommended by the Apifresh best practice guide [17]. Drying under natural conditions, however, should be avoided, as this can promote microbial contamination [23].

Reducing the water content of bee pollen has negative effects on its sensory characteristics and its nutritional value, such as loss of vitamin E, β-carotene and pro-vitamin A [29]. Dominguez-Valhondo et al. [30] reported that freeze-drying

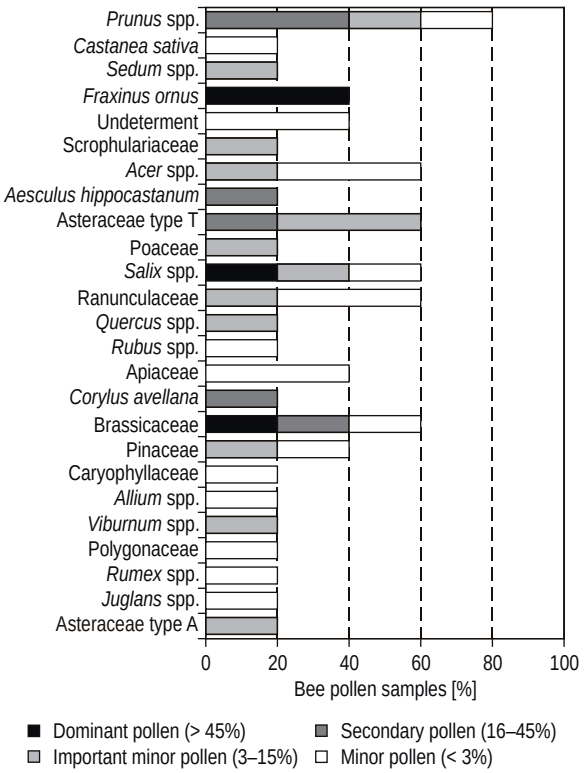

Fig. 1. Botanical origin of bee pollen samples collected in April (n = 5).

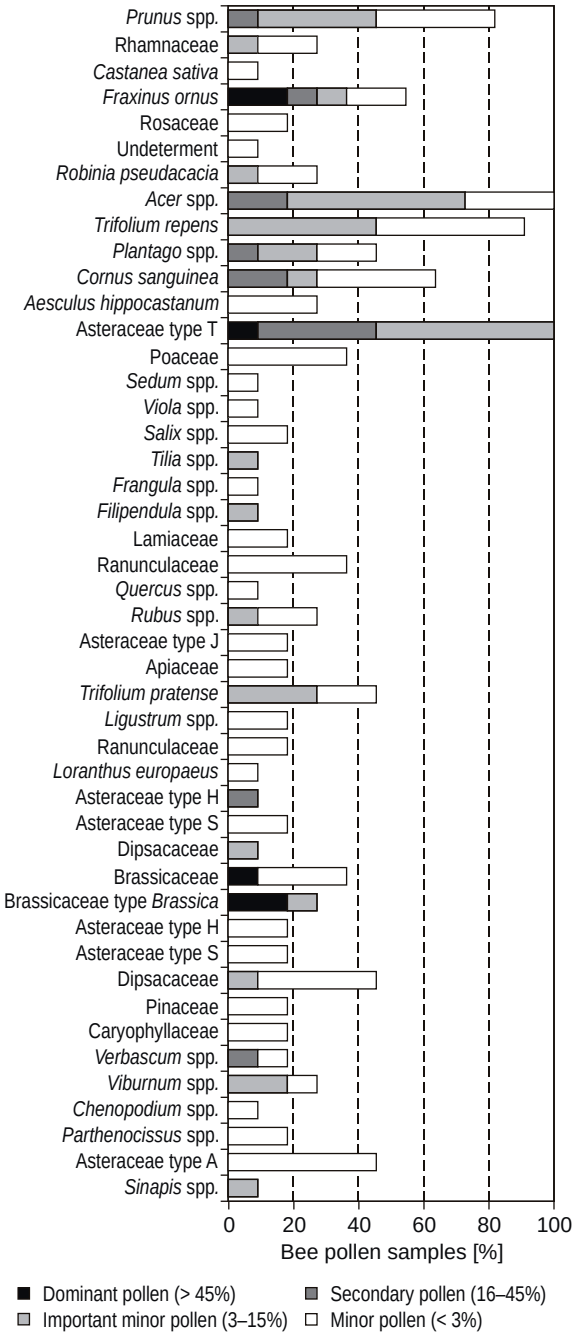

Fig. 2. Botanical origin of bee pollen samples collected in May (n = 11).

is the best way to preserve the chemical and biological properties of bee pollen. However, consumers are showing more interest in fresh bee pollen, as it has a better preserved chemical, biological and sensory properties, as well as the visual aspect, aroma, taste and texture. For consumption, fresh bee pollen also dissolves better [17].

Most previous studies indicated the water content of dried bee pollen, and so these data on FW basis are difficult to compare because of this lack of information on the water content of fresh bee pollen. In Slovenian bee pollen, the average fresh pollen water content was particularly high, so there was a risk of microbial contamination. This indicates the need for hygienic guidelines for the process of producing the bee pollen, with an appropriate method for distribution of the fresh bee pollen. The water content in these fresh Slovenian bee pollens were generally higher than those reported by HUMAN and NICOLSON [7], and NICOLSON and HUMAN [31] for pollen samples of *Aloe greatheadii* var. *davyana* and sunflower (*Helianthus annuus* L., Asteraceae) from South Africa (188.0 g·kg^{-1}, 197.8 g·kg^{-1}, respectively). The differences are probably due to the different climate conditions. The water content in dried bee pollen from Brazil was reported to range from 16.9 g·kg^{-1} to 78.4 g·kg^{-1} [32], from China 18.2–73.3 g·kg^{-1} [22], and from Spain 43.3–66.7 g·kg^{-1} [23].

The water content is an important criterion of bee pollen quality. In the proposal of CAMPOS et al. [3], dry bee pollen for human consumption should contain 60.0 g·kg^{-1} to 80.0 g·kg^{-1} water. An excessive water content (i.e. > 100 g·kg^{-1}) may result in microbial contamination, mainly by moulds and yeasts [2, 23].

The protein content of bee pollen depends on

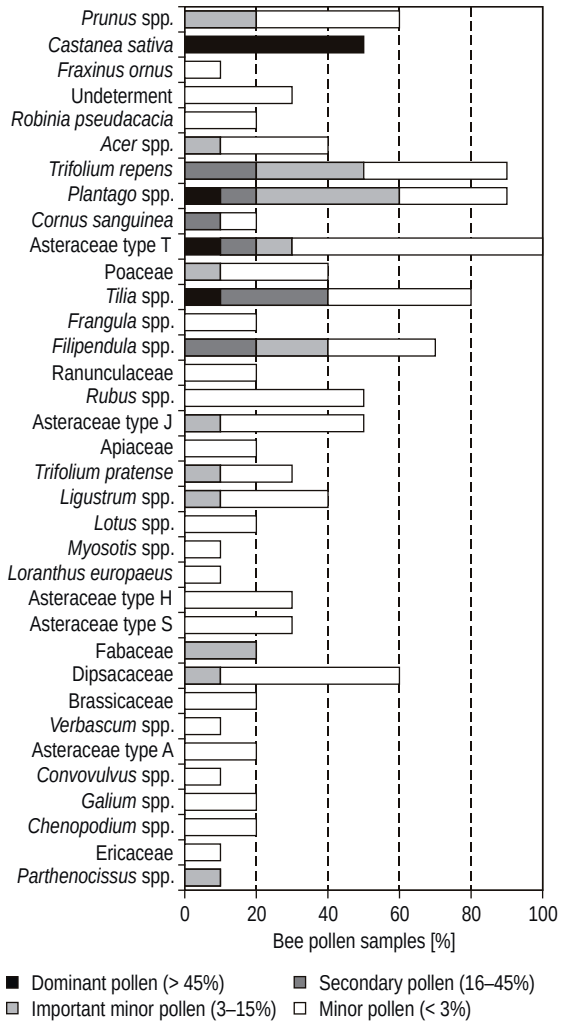

Fig. 3. Botanical origin of bee pollen samples collected in June (n = 10).

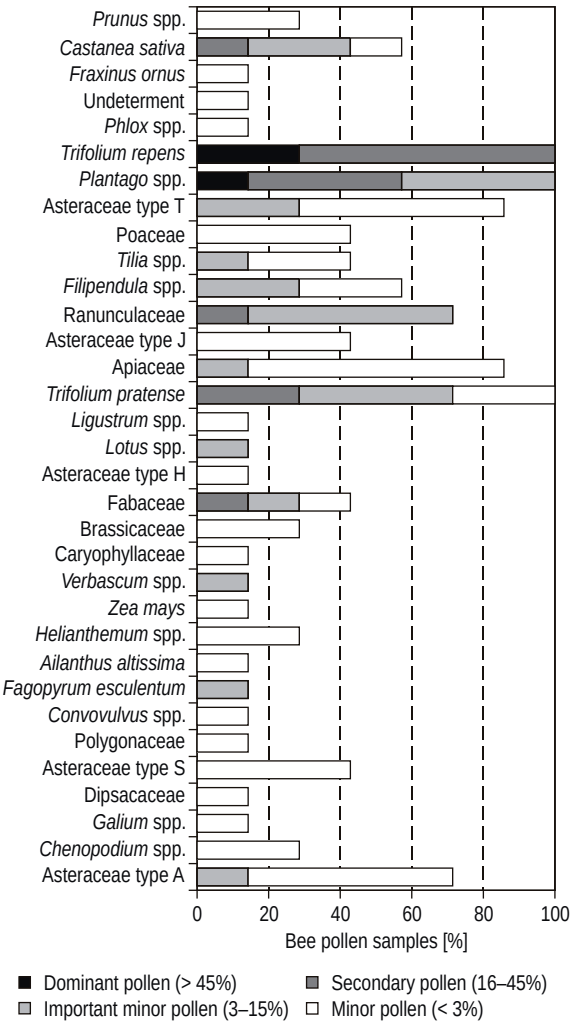

Fig. 4. Botanical origin of bee pollen samples collected in July (n = 7).

Tab. 1. Chemical composition of the Slovenian bee pollen samples.

Parameter	Fresh weight				Dry weight			
	Mean	Minimum	Maximum	± *SD*	Mean	Minimum	Maximum	± *SD*
Water [g·kg^{-1}]	227.3	157.0	292.0	37.3	-	-	-	-
Protein [g·kg^{-1}]	174.6	130.0	229.0	26.4	227.3	160.3	323.4	41.2
Fat [g·kg^{-1}]	73.6	45.0	123.0	15.7	95.5	60.7	157.9	21.1
Ash [g·kg^{-1}]	20.6	13.0	28.0	3.8	26.7	16.5	38.8	4.8
Total carbohydrate [g·kg^{-1}]	503.8	392.7	600.0	56.7	650.5	547.5	739.8	48.5
Energy [MJ·kg^{-1}]	14.3	13.0	15.4	0.7	18.5	17.8	19.8	0.5

Number of bee pollen samples analysed was $n = 32$. *SD* – standard deviation.

the botanical source [3], and this is regarded as a reliable direct measurement of its nutritional value [33, 34]. The pollen collected from plants by bees can have a protein content from 120.0 g·kg^{-1} to 610.0 g·kg^{-1} [35]. In the fresh Slovenia bee pollens, the protein content varied from 130.0 g·kg^{-1} to 229.0 g·kg^{-1}; on a DW basis, this represented 160.3–323.4 g·kg^{-1} (Tab. 1). These data are similar to those reported by Feas et al. [36] for bee pollen from Portugal (191.0–271.0 g·kg^{-1} DW), and a little higher than those reported by Yang et al. [22] from China (142.4–289.5 g·kg^{-1} DW). Human and Nicolson [7] reported that the average protein content for *A. greatheadii* var. *davyana* pollen was 314.0 g·kg^{-1} DW, and for sunflower (*H. annuus*), 142.1 g·kg^{-1} DW [31]. However, Estevinho et al. [23] reported generally higher protein contents (242.3–341.8 g·kg^{-1}) in dried bee pollen from Portugal, and Nogueira et al. [37] reported generally lower values from Portugal and Spain (125.0–251.5 g·kg^{-1}). Almeida-Muradian et al. [1] reported that the mean protein content in dried bee pollen from Brazil is 200.0 g·kg^{-1} and Carpes et al. [32] reported values from 150.4–276.9 g·kg^{-1}. These differences are probably due to the different plant sources and the different environmental conditions [1, 3].

The fat content of the fresh bee pollen samples from Slovenia varied from 45.0 g·kg^{-1} to 123.0 g·kg^{-1}, and from 60.7 g·kg^{-1} to 157.9 g·kg^{-1} on DW basis. The data for the fat content on DW basis are higher than those reported by Yang et al. [22] for China (6.6–107.9 g·kg^{-1} DW), and by Feas et al. [36] for Portugal (43.0–63.0 g·kg^{-1} DW). In the many reports on fat content in dried bee pollen, the data varied from 10.0 g·kg^{-1} to 130.0 g·kg^{-1}, with significant differences seen in relation to the botanical origin [3]. This range includes the fat content reported by Human and Nicolson [7] and Nicolson and Human [31] for bee pollen samples of *A. greatheadii* var. *davyana* and *H. annuus* from South Africa (55.0 g·kg^{-1} DW). Nogueira et al. [37] reported values from 23.5 g·kg^{-1} to 33.3 g·kg^{-1} for dried bee pollen from Portugal and Spain, and Estevinho et al. [23] reported values from 23.3 g·kg^{-1} to 33.2 g·kg^{-1} for bee pollen samples from different regions in Portugal. Similar ranges of fat content were reported for bee pollen by Carpes et al. [32] and Soares de Arruda et al. [24] for Brazil (37.0–65.0 g·kg^{-1}, 53.9 g·kg^{-1}, respectively). However, Almeida-Muradian et al. [1] reported that the average fat content in Brazil dried bee pollen was 60.0 g·kg^{-1}.

The ash content in the Slovenian fresh bee pollens ranged from 13.0 g·kg^{-1} to 28.0 g·kg^{-1}.

Tab. 2. Chemical composition of the bee pollen samples according to the month of collection.

Month	*n*	Water [g·kg^{-1}]	Protein [g·kg^{-1}]	Fat [g·kg^{-1}]	Ash [g·kg^{-1}]	Total carbohydrate [g·kg^{-1}]	Energy value [MJ·kg^{-1}]
April	5	215.0	217.9	101.1^{b}	25.6	655.4	18.6^{b}
May	11	240.0	226.5	110.8^{b}	25.7	637.1	18.8^{b}
June	10	211.0	217.1	77.1^{a}	27.5	678.3	18.1^{a}
July	6	244.0	254.0	93.6^{ab}	28.3	624.5	18.4^{ab}

Values are expressed as grams per kilogram of dry weight. Content of water is expressed as grams per kilogram of fresh weight. Different letters in superscript indicate significant differences within the column (Duncan's test; $\alpha = 0.05$)

This represented, on DW basis, the ash content of 16.5–38.8 g·kg^{-1}, with a mean of 26.7 g·kg^{-1} DW. In China, YANG et al. [22] reported a similar ash content of 17.0–50.1 g·kg^{-1} DW, and in Portugal 20.0–40.0 g·kg^{-1} DW [36]. HUMAN and NICHOLSON [7] reported a mean of 36.0 g·kg^{-1} DW ash content for bee pollen of *A. greatheadii* var. *davyana*, and for the bee pollen from sunflower (*H. annuus*) 16.0 g·kg^{-1} DW [31]. CARPES et al. [32] reported a range of ash contents from 19.0 g·kg^{-1} to 39.1 g·kg^{-1} in dried bee pollen from Brazil. Similar values were reported by SOARES DE ARRUDA et al. [24] and ALMEIDA-MURADIAN [1] for bee pollen from Brazil (27.7–32.4 g·kg^{-1} and 22.0 g·kg^{-1}, respectively). For dried bee pollen from Portugal and Spain, NOGUEIRA et al. [37] reported a range from 5.0 g·kg^{-1} to 31.6 g·kg^{-1}.

The total carbohydrate includes saccharides, starch and dietary fibre, contents of which ranged in the Slovenian fresh bee pollens from 392.7 g·kg^{-1} to 600.0 g·kg^{-1}. On a DW basis, the total carbohydrate content was from 547.5 g·kg^{-1} to 739.8 g·kg^{-1}, with a mean of 650 g·kg^{-1} DW. Again, these data were similar to those reported by FEAS et al. [36] (612.0–706.0 g·kg^{-1} DW). For China, YANG et al. [22] reported from 594.3 g·kg^{-1} DW to 756.4 g·kg^{-1} DW. Also, NICOLSON and HUMAN [31] reported a relatively high mean for the total carbohydrate in bee pollen from sunflower (787.1 g·kg^{-1} DW) and HUMAN and NICOLSON [7] reported a mean of 595.0 g·kg^{-1} DW for the bee pollen of *A. greatheadii* var. *davyana*. ESTEVINHO et al. [23] reported the total carbohydrate of dried bee pollen from Portugal to range from 608.2 g·kg^{-1} to 707.6 g·kg^{-1}. NOGUEIRA et al. [37] reported higher values from Portugal and Spain (696.8–842.5 g·kg^{-1}). In contrast, CARPES et al. [32] reported a lower mean in dried bee pollen from Brazil (521.0 g·kg^{-1}).

The energy values of the fresh bee pollen samples ranged from 13.0 MJ·kg^{-1} to 15.4 MJ·kg^{-1}. On a DW basis, the range was from 17.8 MJ·kg^{-1} to 19.8 MJ·kg^{-1}. These results were different from FEAS et al. [36], who reported lower energy values for bee pollen from Portugal (16.6–17.3 MJ·kg^{-1}), which were similar to those reported for China bee pollen by YANG et al. [22] (16.7–18.7 MJ·kg^{-1} DW). These values were higher than those reported by NOGUEIRA et al. [37] for commercial dried bee pollen from Portugal and Spain (16.8–17.3 MJ·kg^{-1}).

Tab. 2 presents the mean data for these parameters according to the months of the bee pollen collection. The statistical analysis showed that, on DW basis, there were significant differences in the fat content and energy values of the bee pollens between these months. Here, the fat content and energy values for June were significantly lower than those for April and May, but did not differ from those for July. None of the other analysed parameters showed significant differences according to the month of collection. According to a draft for the basic composition requirements for dried bee pollen [3], the data obtained in the present study for the Slovenian bee pollen suit the criteria of the normative for the contents of total protein, fat, ash and total carbohydrate (Tab. 3).

Tab. 3. Draft basic composition requirements for bee pollen (dried), as proposed by CAMPOS et al. [3].

Content of component	International proposals
Water	≤ 6–8%
Total protein	≥ 15%
Total fat	≥ 1.5%
Total ash	≤ 6%
Total carbohydrate	≥ 40%

Sample preparation for detection of free tryptophan

The structure of pollen grains includes two walls, exine and intine, which may cause low digestibility for humans. Both of these walls are indigestible by the human digestive tract, because of the lack of specific enzymes. Only small molecules (e.g. free amino acids) are diffusible through the intine fibrillar structure, and these can be completely lost, as indicated by FRANCHI et al. [38]. The use of pollen in human studies showed that a part of the pollen content is digested and bioavailable, with differences in the degree of digestion according to specific pollen types [38]. Maceration of bee pollen for several hours in water or other liquids is recommended to improve digestibility of bee pollen for humans [8, 39].

For the detection of free Trp in the bee pollen samples in the present study, different extraction conditions and methods for the preparation of the samples were initially tested. Homogenization of bee pollen samples by grinding or crushing them with dry ice did not destruct the pollen grains. This was confirmed under light microscopy, with no changes seen. Extraction with a suitable solvent combined with ultrasound treatment was chosen as an optimal method to obtain amino acids from inside the pollen grains.

The use of MilliQ water as the solvent gave the optimal results, while sodium phosphate buffer (pH 6.75) was also satisfactory. Using methanol, or a combination of methanol and MilliQ water

Tab. 4. Colour, protein and free tryptophan content according to the botanical origin of bee pollen from Slovenia.

Botanical origin	Share [%]	Colour	Protein [g·kg^{-1}]	Free tryptophan [g·kg^{-1}]
Brassicaceae	87	Yellow	246	0.158 ± 0.010^{c}
Prunus pyrus/malus	96	Light yellow	256	0.056 ± 0.005^{ab}
Fagopyrum esculentum	87	Light green	147	0.097 ± 0.045^{b}
Trifolium pratense	93	Yellow-brown	232	0.041 ± 0.007^{a}
Phacelia spp.	96	Violet	251	0.030 ± 0.003^{a}
Asteraceae type A	93	Orange-red	120	0.028 ± 0.004^{a}
Salix spp.	99	Yellow	215	0.197 ± 0.022^{c}
Corylus avellana	99	Yellow	–	0.069 ± 0.008^{ab}

Number of bee pollen samples analysed was $n = 8$. Values are given on fresh weight basis.
Free tryptophan contents are presented as mean ± standard deviation. Different letters in superscript indicate significant differences within the column (Duncan's test; $\alpha = 0.05$)

(10:90, v/v), did not provide good extraction yield. Thus, for good extraction of amino acids from pollen grains, several extractions with MilliQ water combined with ultrasound treatment (for 10 min) were tested.

The content of free Trp in these tested Brassicaceae bee pollen samples decreased after four extractions with MilliQ water at ultrasound treatment. After the first extraction with ultrasound, the content of free Trp was 0.2 g·kg^{-1}. After further three extractions combined with ultrasound, the remaining free Trp detected decreased to 0.04 g·kg^{-1}, where it reamined during further following extraction cycles.

Thus, this extraction of the bee pollen samples in MilliQ water was carried out four times, with 10 min of ultrasound treatment for each extraction. Indeed, ZHANG et al. [14] reported that ultrasound treatment of pollen can increase the amount of free Trp in solution, which is thus confirmed in the present study.

Determination of free tryptophan

The free Trp contents detected in different Slovenian unifloral bee pollen samples in the present study are shown in Tab. 4. The highest free Trp was detected in the bee pollen sample from *Salix* spp. (0.197 g·kg^{-1}). However, the free Trp varied greatly across the pollens of different botanical origin, with those from *Salix* spp. and Brassicaceae being significantly greater than those from the other tested unifloral bee pollens. Overall, from Asteraceae type A to *Salix* spp., the free Trp varied from 0.028 g·kg^{-1} to 0.197 g·kg^{-1}, the free Trp content in the rapeseed pollen (i.e., Brassicaceae; 0.158 g·kg^{-1}) was higher than that reported by ZHANG et al. [14] (0.073 g·kg^{-1}), who also reported the free Trp for *Camellia* and *Lotus* pollen to be 0.068 g·kg^{-1} and 0.067 g·kg^{-1}, respectively. PARAMAS GONZALES et al. [15] reported that the mean content of free Trp in bee pollens was 0.090 g·kg^{-1}. For the *Cistus* and *Echium* pollens analysed, the content of free Trp was under the detection limits [15].

Tryptophan is an essential amino acid that is sometimes lacking in some types of pollen [35, 40]. In the present study, it was shown that Asteraceae type A contained the lowest amounts of free Trp. YANG et al. [22] determined the levels of free Trp for twelve unifloral botanical origins of bee pollen. In comparison with other essential amino acids, the levels of free Trp were greater except for the pollen from *Schisandra chinensis.* The free Trp values ranged from 0.053 g·kg^{-1} (DW) in bee pollen from *S. chinensis*, to 0.443 g·kg^{-1} (DW) in bee pollen from *Rosa rugosa.* Level of free Trp in a study by YANG et al. [22] was also greater than the level determined in the present study for *Fagopyrum esculentum* L. The content of free Trp in this unifloral bee pollen was 0.338 g·kg^{-1} (DW). This discrepancy might be due to differences in the method of determination of free amino acids, and due to other conditions [22, 24]. WEINER et al. [41] reported that free Trp could not be detected in bee pollen from *Campanula trachelium* and *Agrimonia eupatoria.* There were no correlations seen in the present study between the protein content and the free Trp content in the unifloral bee pollen samples from Slovenia ($R^2 = 0.02$).

Trp is one of eight essential amino acids. Therefore it must be supplied in the diet. The major Trp sources include milk and dairy products, meat and sausages, fish, white bread, eggs and potatoes [42]. Possibly because of its low concentra-

tion in the body, which is the lowest of all of the amino acids, Trp may have a rate-limiting role in protein synthesis [41]. The daily requirement estimated by FAO/WHO for Trp intake is 4 mg per kilogram of body weight for adults [43].

Hydrolysis under acidic conditions is necessary when the bound Trp in the protein is determined. Bound Trp is unstable under these conditions. ZANG et al. [14] reported that Trp is present mostly in a bound form. The Trp content in a bound form was greater than in the free form for three types of bee pollens analysed [14]. This was also confirmed in a study by GRÜNFELD et al. [44].

Free amino acids are very important for human nutrition, as they can be immediately used for bodily needs. Sources of Trp are considered to be very important for human diet [3], and the content of Trp represents an added value for this bee product. Thus, following this and previous studies, it is possible to recommend the type of bee pollen that should dominate in any bee pollen mixture, or even to decide on the botanical source according to the consumer needs.

CONCLUSIONS

Bee pollen is not only used as a food, but also as a value-added product that has positive influences on human health. Thus, there is a need for characterization of this bee product to provide consumers with healthy and safe food. As bee pollen is now being used more often in the human diet, and is also a part of various therapies, it is important to establish quality criteria and to standardize the analytical methods. In order to establish the quality criteria, it is necessary to overcome the lack of information about the composition of bee pollens from different countries. Further studies are also needed, especially because of the differences seen for bee pollens according to botanical origin, climate conditions, and nutritional status of the plants. This study is the first that defines the composition of Slovenian bee pollen. For the full characterization of Slovenian bee pollen as a source of important nutrients in the human diet, and also for potential therapeutic purposes, more studies are required. Characterization of bee pollen may increase its economic value. The present study also provides an important contribution to the progress of beekeeping in Slovenia.

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