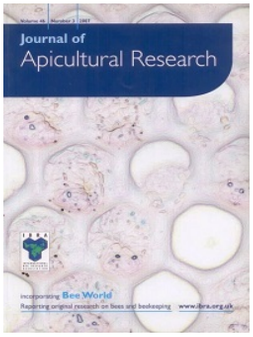

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REVIEW ARTICLE

Pollen composition and standardisation of analytical methods.

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Summary

Honey bee pollen is considered to be a food, and national pollen standards exist in different countries such as Brazil, Bulgaria, Poland and Switzerland. It is the aim of the present work to review pollen composition and the analytical methods used for the evaluation of high quality bee pollen. Based on the experience of different countries and on the results of published research, we propose quality criteria for bee pollen, hoping that in the future they will be used as world wide bee pollen standards.

Keywords: Standardisation of bee products; bee pollen; quality control

Introduction

Pollen is the male gametophyte of flowers. Commercially traded pollen is mainly pollen collected by the honey bee *Apis mellifera* for the purpose of feeding its larvae in the early stages of development. Collected flower pollen is accumulated as corbicular pellets in pollen baskets on the rear legs of the honey bee and it is a mixture of these pellets that comprises bee pollen (Krell, 1996; Campos, 1997; Almeida-Muradian *et al*., 2007). When visiting flowers, bees touch the stamens and their bodies become covered with pollen dust. The bees use their hind legs to compress the pollen into the pollen baskets. The bees moisten the pollen with mouth secretions which help the pollen to cling together and to the basket hairs (Hodges, 1952). These secretions contains different enzymes, e.g. amylase, catalase etc. A pollen load contains up to 10% nectar, which is necessary for packing.

Whilst honey forms the energy source of the bee colony, pollen is the bees' main source of the other important nutrients: proteins, minerals, fats and other substances (Serra-Bonvehi and Escola-Jorda, 1997; Villanueva *et al*., 2002; Bastos *et al*., 2004; Almeida-Muradian *et al*., 2005; Human and Nicolson, 2006). The presence of these compounds cause pollen to be considered as a human food, so national pollen standards exist in a number of countries such as Brazil (Brazil, 2001); Bulgaria (Bulgarian standard 2567111-91); Poland (PN-R-78893 "Obnóza pylkowẽ"- Polish legislation for bee-pollen) and Switzerland (Swiss Food Manual: Pollen Bienenprodukte, BAG -Swiss Federal Office for Public Health). It is the aim of the present work to review the different factors necessary for producing high quality pollen, pollen composition and the analytical methods used. Based on the experience and the research of different countries, general quality criteria for pollen will be proposed, and based on this, our group will be working in the near future on a world wide pollen standard.

A short review of the different quality criteria and on the methods used (analytical methods applied to the quality control of bee-pollen)

Pollen collected by honey bees (bee-pollen) is promoted as a health food with a wide range of nutritional and therapeutic properties (Samochowiec and Wójcicki, 1981; Kosmider *et al*., 1983; Wójcicki *et al*., 1983; Lin *et al*., 1990; Iannuzzi, 1993; Wang *et al*., 1993; Dudov and Starodub, 1994; Liebelt *et al*., 1994;

Yasumoto *et al*., 1995; Bevzo and Grygor'eva, 1997; Campos *et al*., 1997a; Linskens and Jorde, 1997; Bruneton, 1999; Haro *et al*., 2000; Cocan *et al*., 2005; Hamamoto *et al*., 2006; Yamaguchi *et al*., 2006). A high concentration of reducing sugars, essential amino acids, unsaturated and saturated fatty acids, the presence of Zn, Cu, Fe, and high K/Na ratio make honey bee pollen very important for human diets (Serra-Bonvehi and Escola-Jorda, 1997; Villanueva *et al*., 2002; Bastos *et al*., 2004; Almeida-Muradian *et al*., 2005).

The major variable in bee pollen is the floral origin composition of the pollen, which may be affected by differences in catchments area or season (Szczesna *et al*., 2002). Bee pollen, being a mixture of bee collected floral pollens, varies widely in composition. A systematic method for characterising bee pollens in terms of their constituent pollens is needed in view of the growing phytotherapeutic interest in bee pollen products. Campos *et al*. (1996, 1997b) carried out studies involving bee pollen samples collected from different places and years that help in the identification of the floral sources. An approach based on flavonoid/phenolics profiles derived from high performance liquid chromatography has been demonstrated to be more precise and informative than traditional microscopy. This method provides a convenient means for identifying the contributing pollens, and for characterising bee pollens in terms of their predominant constituent pollens. The flavonoid/phenolics profiles obtained in the course of this work also highlighted other observations of interest. For example, bees were shown to be highly selective pollen gatherers from the finding that bee pollens comprise pollen from only a few of the available species. Pollen from only one floral source is usually found in each bee pollen pellet (Campos *et al*., 1996,1997b; Almeida-Muradian *et al*., 2005).

The content of macro- and micronutrients has been published in many papers and the influence of the botanical origin is obvious (Villanueva *et al*., 2002; Serra-Bonvehi and Escola-Jorda, 1997; Almeida-Muradian *et al*., 2005; Bastos *et al*., 2004). Concerning analytical protocols, the majority of authors follow methods described in the AOAC Official Methods of Analysis. These methods can be applied to analyse food, beverage, agricultural, microbiological, pharmaceutical and environmental samples.

Nutrient contents of pollen also change with storage. Szczesna *et al*. (1995a, 1995c, 1995d) estimated the effect of different methods of preservation (freezing, drying at about 40°C and lyophilisation) on selected parameters attributed to the biological quality of bee pollen. Freezing caused no substantial changes in the chemical composition of the pollen loads, so this technique should be recommended when the preservation of the pollen load for nutrition or therapeutic purposes is important. Lyophilisation markedly decreased vitamin C and provitamin A content, but drying at 40°C revealed the most disadvantageous effect. The amounts of four out of nine constituents examined (reducing sugars, total proteins, vitamin C, and provitamin A) markedly decreased. Taking into account the methods of production, these authors offers practical recommendations for the means of preservation and optimum conditions for the storage of pollen loads. Freezing followed by storage at -20°C in pure nitrogen guarantees high biological qualities of bee pollen kept for up to 6 months. Pollen stored for a longer periods should, however, be dried by lyophilisation and stored at -20°C in pure nitrogen to preserve its highest biological activities.

Proposed harmonisation of standard methods and criteria used for the quality control of bee pollen

A bee pollen standard will be valid for pollen as gathered by beekeepers using a pollen trap. This product is generally in the form of pollen loads. From the nutritional point of view, the methods used to analyse the main components of pollen loads; carbohydrates, fats and proteins, should be evaluated using inter laboratory studies and be published as has been done for honey (Bogdanov, 1997). From the hygienic point of view, microbiological safety is the main quality criterion. It is important to control the microbiological quality of pollen, especially the absence of pathogenic germs and fungi following the legislation applied for food. Pollen is the bee product least influenced by contaminants from beekeeping. The main contaminants are heavy metals (Szczesna *et al*., 1993; Jablonski *et al*., 1995; Leita, 1996; Conti and Botre, 2001) and pesticides (Fleche *et al*., 1997; Kubik *et al*., 1999) originating from the environment and from agricultural practices. For optimum quality, pollen should therefore be gathered in areas which are at least 3 km distant from sources of contamination such as heavy traffic or pesticide treated agricultural areas. Requirements for heavy metal content of pollen loads are not more than: Cd – 0.1 mg/kg; Pb – 0.5 mg/kg; As – 0.5 mg/kg, Hg – 0.03 mg/kg.

Another important issue that needs special care is that of mycotoxins that could theoretically develop in bee pollen after mould spoilage (Medina *et al*., 2004) and hence need to be evaluated. The impact of pollens from genetically modified organisms (GMOs) in bee pollen has not yet been evaluated. In the last few years, cultivation of GMOs has increased very rapidly, so the probability of finding bee pollen coming from such plants is higher (Malone and Pham-Delègue, 2001). No published studies have found negative effects of such pollen on human health. In the European Union there is a compulsory requirement (Regulation EC 1829/2003) to label products where the GMO content exceeds 1%, and this could also be applied to pollen once the same value is recommended for honey.

Proposed technical regulation for the identity and quality of bee pollen

Technical regulation for identity

Objective: To establish the identity and the minimum quality requirements for bee pollen.
Target: The regulation will be applied to bee pollen sold in national and international markets.

1. Description

1.1. Definition: Bee pollen is the result of the agglutination of flower pollens, made by worker honey bees, with nectar (and/or honey) and salivary substances, and collected at the hive entrance.
1.2. Classification:
1.2.1. *According to water content*:
1.2.1.1. Bee pollen: The product collected in the original form, with water content between 20-30 %. Storage of such pollen should be in a freezer to avoid bacterial and mould contamination.
1.2.1.2. Desiccated bee pollen: The product submitted to a drying out process in temperatures not higher than 42°C, with water content not higher than 6%.

1.2.2. *According to the floral source content:*
1.2.2.1 Monofloral bee pollen: the major *taxon* need to be not less than 80% (different taxa can be used for specific nutritional and therapeutic purposes).
1.2.2.2 Multifloral bee pollen: include different *taxa*.
1.3. Denomination for sales purposes will include classification according the water and floral source content.

2. Packaging: The product must be packaged in sacks preventing entry of atmospheric moisture, using food grade materials to provide adequate protection. Hygiene practices related to the product preparation must conform to the technical regulation for sanitary conditions and the current GMP (Good Manufacturing Practices) for the food industry. Desiccated bee pollen must be free of mechanical impurities and not be rancid.

3. Additives
Not authorized.

4. Contaminants
No organic or inorganic contaminants may be present in quantities higher than those limited by the specific regulation (this includes no pesticides, antibiotic and/or varroacide residues) (Table 1).

5. Hygiene
5.1. General Considerations:
Hygiene practices related to product preparation must conform to the technical regulation for sanitary conditions and the current GMP (Good Manufacturing Practices) for the food industry.

Table 1. Microbiological and other contaminants of bee pollen.

Microbiological analysis	
Salmonella	Absent / 10 g
Staphylococcus aureus	Absent / 1 g
Enterobacteriaceae	Max.100/g
Escherichia coli	Absent./ g
Total aerobic plate count	<100 000/g
Mould and yeast	< 50 000/g
Organochlorine pesticides	< MRL*
Organophosphate pesticides	< MRL
Pyrethroids	< MRL
Alfatoxin B1	Max. 2 µg/kg
Alfatoxin B1+B2+G1+G2	Max. 4 µg/kg
Cloramphenicol (CAP)	absent
Nitrofuran metabolites	absent
Sulfonamides	absent
Heavy metal Pb	max 0,5 mg/kg
Heavy metal Hg	max 0,01 mg /kg
Heavy metal Cd	max 0,03 mg/kg
Radioactivity (Cs-134 and Cs-137)	<600 Bq / kg

* should be smaller than the values established for honey.

5.2. Macroscopic and Microscopic criteria:
The product must not contain any foreign substances except for the accidental presence of fragments of bees, wood, plants and other material inherent to the harvest process of pollen by the bees. Note: The macro and microscopic tolerance criteria will be further established in a specific regulation.

6. Storage
Fresh, bee collected pollen contains about 20-30 g water per 100 g. This high humidity is an ideal culture medium for micro-organisms like bacteria, yeast and acarid mites (Szczesna *et al*., 1999). For the prevention of spoilage and for preservation of maximum quality the pollen must therefore be harvested daily and immediately placed in a freezer. After thawing, pollen may be kept for only a few hours and should be further processed as soon as possible. After drying, the water content should be 4-8g per 100 g pollen. Under these conditions, pollen retains its quality for a storage period of two years if stored in a cool, dry, dark place (Szczesna *et al*., 1999).

7. Microbial quality
The microbiological content should correspond to the hygienic standards. The European Union standard for microbiological quality follows the A.O.A.C. methods and levels (Table 1).

8. Labelling
Each package of bee pollen must be labelled with: denomination of contents that includes the classification of the product according to: the water content and, if necessary for a specific purpose, the floral content; the main nutrients (carbohydrates, fats and proteins), locality of harvesting, date limit for consumption, name of the producer, and batch number. The producer will need to retain samples of each lot for control supervision by the authorized governmental authorities. Also required will be: the date of preparation, year of production, weight (gross, tare, net), and the address of the producer/packer. Additional information such as the content of vitamins, polyphenols, minerals, free sugars, unsaturated fat acids (w3 and w6) and free amino acids should complement the label, improving the value of the product. Packages must carry the label "Not for consumption by infants under 1 year old", as cases of "Sudden infant death" could theoretically be caused by the bacterial spores of *Clostridium botulinum* and to allergic proteins which can be found in bee pollen.

9. Analytical methods for quality control:
Will be included in the regulation.

Quality criteria

Organoleptic characteristics

1. Sensory analysis

Colour, appearance, odour and taste vary according to the botanical origin.

Colour: varies from white to black, mostly yellow, orange or yellow-brown, but many different colours are possible according to the floral sources (Hodges, 1952; Jablonski *et al*., 1995).

Appearance: as so called "pollen loads", heterogeneous grains, with different shapes and sizes, mainly spherical.

Odour: typical for pollen loads, specific according to floral sources

Taste: specific, sweet, sour, bitter, spicy.

Identity: microscopical ascertainment of presence of only pollen loads

Defects: off-odour and taste, moulds, fermented, rancid, visual impurities.

2. Microscopical examination

The pollen should not contain: acaro-entomological contaminations (live or dead insects), larvae or eggs, impurities such as dead bees (workers, brood, pieces of their bodies), propolis, wax, plant particles or other foreign matter such as soil, sand, etc. Pollen analysis can be used for the determination of the botanical origin (Moreti *et al*., 2002; Bastos *et al*., 2004; Almeida-Muradian *et al*., 2005) but HPLC/UV can also be used for taxa identification and this data can included the % of the major *taxon* in the sample (Campos *et al*., 1997b). The same methodology as used for pollen analysis of honey can also be applied (Louveaux, *et al*., 1978).

3. Composition

Bee pollen is composed of proteins, lipids, sugars, fibre, mineral salts, amino acids, phenolic compounds and vitamins. A high concentration of reducing sugars, essential amino acids and unsaturated/saturated fatty acids, the presence of Zn, Cu, Fe, and high K/Na ratio make honey bee pollen very important for human diets (Campos *et al*., 1997a). Pollen composition is very variable depending on the floral origin, as shown in Table 2. Draft basic composition requirements are given in Table 3 as a proposal.

3.1. Analytical methods

Physico-chemical characteristics:

Water content

The determination of pollen water content is carried out after drying to a constant weight in a cabinet dryer, infra-red balance (Oliveira, 2006) or by the Karl-Fischer method (Serra-Bonvehi and Marti Casanova, 1987; Gergen, *et al*., 2006). Some countries have established minimal requirements for dried pollen: Brazil: max. 4 g/100 g; Switzerland, Poland: max. 6 g/100 g; Uruguay: max. 8 g/100 g; Bulgaria: max. 10 g/100 g.

Carbohydrates

These are one of the main components. They are mainly polysaccharides such as starch and cell wall material (Stanley and Linskens, 1974; Talpay, 1984). Generally the carbohydrate content will be calculated: 100 less the sum of water, fat and protein content. The calculated carbohydrate content will be greater than that determined by analytical methods (GC, HPLC). The reason for this is that a part of the carbohydrate is composed of crude fibre and cell wall material, which are generally not determined by chemical methods, but are determined by calculation. The sugars fructose (F), glucose (G) and sucrose comprise about 90 % of all low molecular sugars (Serra-Bonvehi *et al*., 1986), while the ratio of the different sugars differs from plant to plant (Solberg and Remedios, 1980; Szczesna *et al*., 2002). The F/G ratio varies between 1.0 and 2.5 (Szczesna *et al*., 2002).

Crude fibre

Bell *et al*. (1983) found values between 7 and 20 g/100g, but the variation between the minimum and the maximum value is rather large, due probably to the different methods used (Herbert and Shimanuki, 1978; Solberg and Remedios, 1980; Bell *et al*., 1983; Talpay, 1984; Serra-Bonvehi *et al*., 1986).

Proteins and amino acids

The protein content of pollen varies greatly, depending on the botanical origin. Only about 1/10 of the total protein comes from free amino acids. Protein content is a standard determination of %N by the Kjedahl method, using a conversion factor of 6.25 or 5.6 (Rabie *et al*., 1983). For calculating the protein content of pollen loads we recommend the use of N x 5.6 rather than N x 6.25 (Rabie *et al*, 1983). This factor has also been used by other authors. Seventeen different amino acids may be present in pollen loads. Proline, glutamic and aspartic acids, lysine and leucine are the predominant amino acids, constituting approximately 55% of total amino acids (Szczesna *et al*, 1995b; Szczesna and Rybak-Chmielewska, 1998).

Nowadays tryptophan sources are considered very important for reducing depression and anxiety. Tryptophan levels could perhaps be used to improve the importance of the product. This is also valid for phenylalanine. It is important to check for certain pollen protein allergens (Rimpler, 2003).

Lipids

There are considerable differences in the fat composition of bee pollen, depending on the botanical origin (Szczesna *et al*., 1995b; Szczesna and Rybak-Chmielewska, 1998).

There are mainly polar and neutral fats (mono-, di and triglycerides), as well as small amounts of fatty acids, sterines and hydrocarbons (Serra-Bonvehi *et al*., 1986). Results from GC analysis show that the lipids in the extract consist mainly of: linolenic, palmitic, linoleic and oleic acids. Unsaturated fatty acids constitute on average about 70% of the total (Serra-Bonvehi and Escola-Jorda, 1997; Szczesna and Rybak-Chmielewska, 1998). Bastos *et al*. (2004) also found oleic acid, linoleic, araquidic and palmitic, 19-56% total unsaturated acids.

Minerals and trace elements

There is considerable variation depending on the pollen type. Determination is carried out on pollen ash, most frequently by atomic absorption. The main mineral is K (about 60% of total mineral content), Mg constitutes about 20%, and Na and Ca 10% (Szczesna and Rybak-Chmielewska, 1998).

Table 2. Detailed composition of bee pollen (dried).

Main components	**Content Min – Max g/100g dry weight**	**References**
Proteins	10-40	Herbert and Shimanuki, 1978; Solberg and Remedios, 1980; Bell *et al*., 1983; Talpay, 1984; Serra- Bonvehi *et al*., 1986; Szczesna *et al*., 1995b; Szczesna and Rybak-Chmielewska 1998; Almeida-Muradian *et al*, 2005
Lipids	1-13	Stanley and Linskens, 1974; Herbert and Shimanuki, 1978; Solberg and Remedios, 1980; Bell *et al*., 1983; Talpay, 1984; Serra- Bonvehi *et al*., 1986; Szczesna *et al*., 1995b;Szczesna and Rybak-Chmielewska 1998; Almeida-Muradian *et al*, 2005
total carbohydrates*	13-55	Stanley and Linskens, 1974; Szczesna *et al*., 1995b; Szczesna and Rybak-Chmielewska 1998; Szczesna *et al*., 2002; Bogdanov, 2004
Dietary fibre, pectin	0,3-20	Bell *et al*., 1983
Ash	2-6	Stanley and Linskens, 1974; Bell *et al*., 1983; Talpay, 1984; Serra-Bonvehi *et al*., 1986; Szczesna *et al*., 1995b; Szczesna and Rybak-Chmielewska 1998; Almeida-Muradian *et al*, 2005
undetermined	2-5	Bell *et al*., 1983

Minerals, trace elements	**mg/kg**	**References**
Potassium	4000-20000	Stanley and Linskens, 1974;Herbert and Shimanuki, 1978; Serra-Bonvehi *et al*., 1986
Magnesium	200-3000	idem
Calcium	200-3000	idem
Phosphorus	800-6000	idem
Iron	11-170	idem
Zink	30-250	idem
Copper	2-16	idem
Manganese	20-110	idem

Vitamins	**mg/kg**	**References**
β–Carotene	10-200	Talpay, 1984; Oliviera, 2006
B_1; Thiamin	6-13	Stanley and Linskens, 1974; Szczesna and Rybak-Chmielewska 1998;
B_2; Riboflavin	6-20	idem
B_3; Niacin	40-110	idem
B_5; Pantothenic acid	5-20	idem
B_6; Pyridoxin	2-7	idem
C; Ascorbic acid	70-560	Talpay, 1984; Oliviera, 2006
H; Biotin	0,5-0,7	Stanley and Linskens, 1974; Szczesna and Rybak-Chmielewska 1998;
Folic acid	3-10	idem
E; Tocopherol	40-320	Oliviera 2006

* Carbohydrates calculated after determinations of proteins and lipids .

Table 3. Draft basic composition requirements for bee pollen (dried).

Component		Content
Water content	not more than	6-8 g/100 g *
Ash content		
Total	not more than	6 g/100 g
not dissolved in 10% HCl	not more than	0.3 g/100 g
Total protein content (N x 6.25)	not less than	15 g/100 g
Sugar content (total)	not less than	40 g/100 g
Fat	not less than	1,5 g/100 g

*the suggested range is 6-8 %. The maximum humidity can only be established after future harmonisation studies, described in the text.

Vitamins and other biologically active compounds
Pollen contains different vitamins (see Table 2) and also polyphenolic compounds (Tomas-Barberan *et al*., 1989; Szczesna *et al*., 1991; Campos *et al*., 1997b; Campos *et al*., 2003). The polyphenolic content of pollen can include derivatives of phenolic acids and flavonoids that are species specific (2-5% w/w).

Contaminants
At present there are no specific limits (MRL) for contaminants in pollen. The values given in Table 3 are maximum levels found in the literature. As with honey, no antibiotics should be present in pollen as these are forbidden for use in the EU. In general, it seems that bacterial contamination is a greater problem than pesticide, antibiotic or heavy metal contamination (Bogdanov, 2006).

Draft standard for pollen composition
Based on the different national standards, we propose a "draft standard" for pollen composition. We are aware that this draft may be subject to change. The most sensitive point from a quality point of view is the humidity of pollen. Very dry pollen (less than 5 %) is less attractive from sensory point of view than more moist pollen, with 7–10 % humidity. The differences might in part be due to the use of different methods of determination. National requirements vary from 4 to 10 %. Thus the proposed range with a maximum of 6 to 8 % is only tentative, and the optimum value can only be established in the future. An international standard can only be established when pollen from different countries is tested with established harmonised methods. There is therefore some way to go until a world pollen standard can be established.

Conclusions

From all the research results presented in this paper, and some quality requirements for pollen from different countries (Bulgarian Standard 2567111-91; Swiss Food Manual: Pollen Bienenprodukte, BAG -Swiss Federal Office for Public Health; Brazil: Instrução Normativa n.3, de 19 de Janeiro de 2001. Regulamentos Técnicos de Identidade e Qualidade de apitoxina, cera de abelha, geléia real, geléia real liofilizada, pólen apícola, própolis e extrato de própolis; Poland: PN-R-78893 "Obnóza pylkowe"- Polish legislation for bee-pollen) it is clear that there is already considerable knowledge in this field. The next steps to be carried out in the future will include: validation of the methods using inter laboratory studies; application of the methods on real samples products in order to see how these standards apply; and establishment of a standard for pollen composition requirements.

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