



Determination of water content in Brazilian honeybee-collected pollen by Karl Fischer titration

Marcelo A. Morgano^{a,*}, Raquel F. Milani^a, Márcia C.T. Martins^{b,c}, Delia B. Rodriguez-Amaya^c

^a Food Science and Quality Center, Institute of Food Technology, Av. Brasil 2880, Campinas, SP 13070-178, Brazil

^b Adventist University of São Paulo, Estr. Itapeperica 5898, São Paulo, SP 05858-001, Brazil

^c Faculty of Food Engineering, State University of Campinas, CP 6121, Campinas, SP 13083-862, Brazil

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ABSTRACT

This paper assesses the performance of a chemical method based on the Karl Fischer titration to determine the water content in samples of dehydrated honeybee-collected pollen. The following analysis parameters were investigated: extraction temperature, particle size, reaction time, and weight of a dried pollen sample. After optimization, the method was used to determine the water content of 154 samples of dried honeybee-collected pollen from different geographical regions of Brazil. The Karl Fischer titration method, performed using a solvent mixture of methanol and n-octanol (1:1 v/v) at 50 °C on pollen particles 600 µm in size produced the best results. Mean values for water content of the 154 samples of dried honeybee-collected pollen from 12 Brazilian regions ranged from 3% to 9%.

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1. Introduction

Bee pollen has been a part of the human diet due to its high nutritional value and consists of approximately 40% carbohydrates, 35% proteins, 4–10% water, 5% lipids, and 5–15% other substances, such as amino acids, vitamins, minerals, antibiotics and antioxidant substances (Bonvehi & Casanova, 1987; Loper, Standifer, Thompson & Gilliam, 1980; Louveaux, 1988). It is popularly believed that regular consumption of bee pollen has a beneficial effect on several medical conditions, such as: depression, anemia, stress-related diseases, memory loss, intestinal and prostate problems, impotence, ageing, impaired immune functions, among others (Linskens & Jorde, 1997; Masson, 1994).

The water content of bee pollen is an important quality parameter or indicator, as it directly influences several basic characteristics of the product, in addition to directly affecting the product's keeping quality and typical flavor, as well as product and aroma preservation. A high water content may increase the activity of microorganisms and enzymes, which in turn, may change sensory characteristics of the product. On the other hand, a much reduced water content may result in rapid rancidity (Morgano, Faria, Ferrão, Bragagnolo & Ferreira, 2008).

Among the risks of consuming a nutritionally valuable food with high water activity and commonly stored at room temperature, one is contamination by fungi, many of which produce carcinogenic mycotoxins. Therefore, bee pollen must be subjected to a drying process to reduce its water content. Bee pollen dehydration demands expertise, practice and appropriate equipment, and should be performed taking special care and precautions to prevent sensitive and/or labile constituents from degrading, thereby ensuring both the integrity of its components, as well as its biological properties (Jodral, Fernández, Bentabol & Liñán, 1992).

Gravimetric methods are still the commonest test options for determining the water content in pollen samples, but it has one major disadvantage in that it is excessively time-consuming. Heinze & Isengard (2001) consider that the gravimetric method is not the most appropriate to determine the water content in some food systems, since the existing water may be strongly linked to polar organic compounds. The high temperatures necessary to liberate this water may result in degradation reactions which lead to the formation of volatile compounds. In addition, volatilization of substances that gives flavor and taste to food may also occur.

According to Isengard, Schultheiß, Radovic & Anklam (2001) and Isengard (2001), the Karl Fischer titration may be used as a reference method to determine the water content in products with a complex composition, such as bee pollen, provided that it is preceded by experiments to determine optimal conditions for analysis, such as the most appropriate extraction temperature, the best solvent to dissolve the sample, and the particle size of the

* Corresponding author. Tel.: +55 19 37431778; fax: +55 19 32424585.

E-mail address: morgano@ital.sp.gov.br (M.A. Morgano).

sample (Gergen, Radu, Bordean & Isengard, 2006). The major advantages to this method are: measurements performed in a few minutes, minimum sample preparation, and no loss of volatile compounds (Bastos, Rocha, Cunha, Carvalho & Torres 2006; Bonvehi & Casanova, 1987; Gergen et al., 2006). Bonvehi & Jordá (1997) emphasize that the outstanding accuracy of the Karl Fischer method makes it as the obvious method of choice for the determination of the moisture level in honeybee-collected pollen.

The technical regulation on the identity and quality of Brazilian bee pollen (BRASIL, 2001, pp. 18–23) defines dehydrated bee pollen as the product subjected to a drying process at temperatures above 42 °C and with a water content smaller than 4%. In Argentinean and Uruguayan legislation, the latter value is set to 8% (Campos et al., 2008; Krell, 1996, pp. 87–113). Other countries also have less strict limits on water content: in Switzerland and Poland, the maximum value is 6% and in Bulgaria 10% (Campos et al., 2008).

The aim of this article was to evaluate the conditions for the determination of water content in samples of dried honeybee-collected pollen by the Karl Fischer titration, such as extraction temperature, particle size, reaction time, and weight of the dehydrated pollen sample to determine the water content in pollen samples collected from different Brazilian regions.

2. Materials and methods

2.1. Samples

In this study, 154 samples of dried honeybee-collected pollen from 11 Brazilian states and the Federal District (Table 1) were obtained directly from the producers. Samples (200–300 g) were sent in mostly contained in plastic bags and, to a lesser extent, in glass bottles. Most samples had been collected in the same month they were received for analysis.

Every sample was granulated. Immediately after receiving the samples, they were vacuum-sealed in polyamide/polyethylene film to prevent them from absorbing moisture and oxygen from the atmosphere and subsequently stored in a freezer at –16 °C until analysis. Before analysis, the samples were quartered with a stainless steel-quartering device and then grounded in a refrigerated IKA Labortechnik M20 Universal mill (Staufen, Baden, Germany) fitted with a tungsten helix. Next, the samples were sieved – depending on the experiment – to 20-mesh (850 µm), 25-mesh (710 µm) and 30-mesh (600 µm) to standardize the size of the pollen particles.

Table 1
Origin of 154 dried honeybee-collected bee pollen samples from different regions in Brazil.

State	Location	Total samples
Bahia	Canavieiras, North Bahia and Ilhéus	37
Ceará	Trairi	1
Federal District	Brasília	3
Espírito Santo	Mountain Region	10
Minas Gerais	Marmelópolis and Belo Horizonte	10
Piauí	Teresina	1
Paraná	Ilha Grande	10
Rio Grande do Sul	Cruz Alta	9
Santa Catarina	Campos Novos and Porto União	30
Sergipe	Neópolis, Salgado and Aracaju	18
São Paulo	S. J. dos Campos, Santa Branca, Holambra and Guaratinguetá	23
Mato Grosso	Cárceles	2

Table 2

Influence of extraction temperature on water content determination by the Karl Fischer titration method, using methanol and n-octanol 1:1 (v/v) as solvent (weight range of the pollen samples: 70–80 mg; particle size: 600 µm).

Temperature (°C)	Water content ± standard deviation (%)	CV* (%)	Mean reaction time (s)
27 (Ambient)	6.34 ± 0.12 ^a	1.89	457
30	6.26 ± 0.22 ^a	3.47	426
50	6.35 ± 0.10 ^a	1.55	169

*CV = Coefficient of variation of 5 repetitions.

^a Comparison of treatments with varying extraction temperature by the Tukey–Kramer test. Values followed by the same letter do not significantly differ at the 5% level.

2.2. Karl Fischer titration

Karl Fischer titration was performed according to the method proposed by Gergen et al. (2006), using an automatic METROHM Titrino 785 titrator (Herisau, Switzerland). Titrations were performed using apura Titrant 5 (Merck) as the titrant and a mixture of methanol p.a. (Merck) and n-octanol alcohol p.a. (Vetec) 1:1 (v/v) as solvents. The polarization stream for potentiometric determination of reaction endpoint was 10 µA and titration endpoint voltage was 100 mV. Karl Fischer titration was performed using a jacketed reaction vessel with temperature adjusted and kept constant by a thermostatically heated water bath.

2.3. Water content determination – Gravimetric method (Horwitz, 2005)

2000 g of pollen sample were transferred to tared aluminum capsules, weighed and placed in a vacuum oven at a temperature of 70 °C for 8–10 h, until constant weight.

2.4. Statistical analyses

In order to verify if the means of water content obtained for the dehydrated bee pollen samples from different treatments were statistically different at $p < 0.05$, the Tukey–Kramer multiple comparison test (Vieira, 1981) and analysis of variance was applied using the software Statistica software program, version 5.5 (StatSoft Inc., Tulsa, USA).

3. Results and discussion

The influence of temperature, particle size, reaction time, and weight of dehydrated pollen samples on the determination of water content was evaluated using the Karl Fischer titration method (Tables 2 and 3). It was observed that the average reaction time

Table 3

Influence of the weight of pollen samples on the determination of the water content determination by the Karl Fischer titration method, using methanol and n-octanol 1:1 (v/v) as solvent; particle size of 600 µm, at temperature of 50 °C ($n = 10$).

Weight ± standard deviation (mg)	Water content ± standard deviation (%)	CV (%)
50 ± 4	6.33 ± 0.13 ^a	2.07
74 ± 2	6.35 ± 0.10 ^a	1.55
100 ± 3	6.43 ± 0.12 ^a	1.91
150 ± 5	6.52 ± 0.10 ^a	1.39

CV = Coefficient of variation of 6 repetitions.

^a Comparison of treatments with varying weighing sample weights by the Tukey–Kramer test. Values followed by the same letter do not significantly differ at the 5% level.

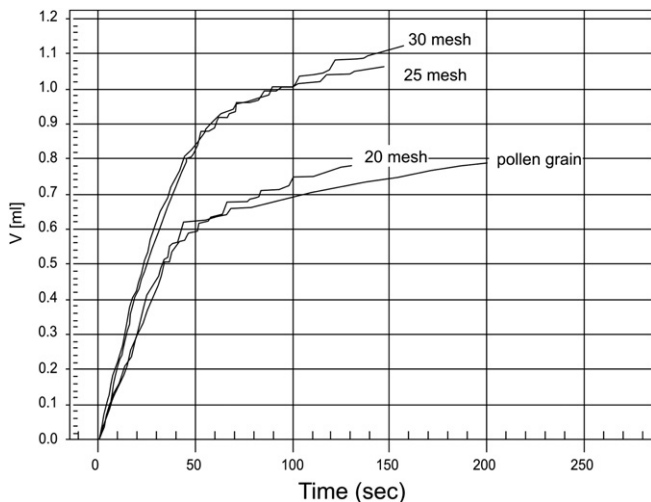


Fig. 1. Influence of the particle size of honeybee-collected pollen on the Karl Fischer titration curve.

reduces as temperature increases. The influence of the temperature is shown in Table 2.

The statistical test results comparing the mean values for water content at varying reaction temperatures are depicted in Table 2. The mean values obtained for the entire temperature range investigated (27–50 °C) were not statistically different at the 95% level of confidence. The results also showed that a rise in temperature results in lower coefficients of variation between replicates.

Gergen et al. (2006) reported a positive influence of temperature on bee pollen water extraction using Karl Fischer titration. These authors found higher values for water content in samples tested at 50 °C compared with samples tested at 26.5 °C. However, in our study, a gradual increase in the water contents of samples tested at 27 °C and 50 °C was not observed. Nevertheless, the average titration time was shorter at 50 °C, with the values for water content remaining unchanged. This finding confirms 50 °C as the temperature of choice for the determination of the water content in bee pollen.

Results of the water content determined in bee pollen samples with different weights (ranging from 50 to 150 mg) are presented in Table 3. The Tukey–Kramer multiple comparison test showed that there was no statistical difference between the mean values for water contents at the 5% error level. A sample weight between 74 and 100 mg was selected to determine the water content of 154 bee

pollen samples. The values obtained for water content are very close to and consistent with the values obtained by the conventional gravimetric method in a vacuum oven at a temperature of 70 °C ($6.4 \pm 0.1\%$, $n = 5$).

Fig. 1 shows the influence of particle size on the titration curve. Bee pollen of the same sample but of different mesh size (original grain, particles passed through 20-mesh (850 μm), 25-mesh (710 μm) and 30-mesh (600 μm) sieves), results in the differences in the titration curve, indicating that controlling particle size is important to determine the water content in dehydrated bee pollen samples. Pollen particles smaller than 600 μm are known to have strong electrostatic attraction, capable of seriously interfering with the accuracy of the analysis and thus were not tested.

Our results confirm those reported by Gergen et al. (2006), who also noted a strong influence of particle size on the result of water extraction tests performed on pollen grains. However, these authors did not standardize the particle size by passing the bee pollen sample material through standard sieves of various sizes, performing tests instead on samples containing particles of smaller size than the particle size of bee pollen ground with mortar and pestle. The values of these were then compared with the values of mill-ground and unground samples, respectively. In this study, the samples were grounded in a mill and then sieved to accelerate and standardize the process. This procedure also allowed to select the most appropriate mesh size for the determination of the water content in bee pollen: 30-mesh (600 μm).

The intermediate accuracy of the Karl Fischer titration method was determined on two different days by subjecting 10 replicate samples – particle size of bee pollen to significantly differs particle size of 30-mesh (600 μm) and weighing between 74 mg and 100 mg – to analysis each day, at a temperature of 50 °C and using a mixture of methanol and n-octanol 1:1 (v/v) as solvent. The results obtained were very similar: 1st Day = $6.33 \pm 0.13\%$; 2nd Day = $6.35 \pm 0.10\%$, and did not show any statistically significant difference at the 5% error level.

The results achieved with Karl Fischer titration on samples weighing between 74 and 100 mg ($6.35 \pm 0.13\%$, $n = 10$; $6.43 \pm 0.12\%$, $n = 6$, respectively) were comparable to the values obtained by the gravimetric method ($6.40 \pm 0.07\%$, $n = 5$). The Tukey–Kramer multiple comparison test did not reveal any significant difference between the means of the two methods (gravimetric and Karl Fischer titration) at the 5% level of significance.

Table 4 presents the water content values of 154 samples of dehydrated bee pollen from several Brazilian producing regions, analyzed in triplicate. The water content values show that the

Table 4
Mean water contents \pm estimated standard deviation (SD), minimum value, maximum value, median and number of samples with water contents higher than 4% of a total of 154 samples of dried honeybee-collected pollen from different producing regions in Brazil, collected from 2007 to 2008 ($N =$ number of samples analyzed per state).

Sample Origin	N	Water content (%)				H > 4%*
		Mean \pm SD	Minimum value	Maximum value	Median	
Bahia	37	6.12 \pm 0.01	3.32	7.63	6.37	36
Ceará	1	5.48	–	–	–	1
Federal District	3	5.60 \pm 0.01	5.00	6.49	5.31	3
Espírito Santo	10	5.48 \pm 0.01	4.61	6.25	5.44	10
Minas Gerais	10	5.18 \pm 0.01	3.00	6.65	5.37	9
Piauí	1	5.69	–	–	–	1
Paraná	10	5.14 \pm 0.01	3.30	6.72	4.83	9
Rio Grande do Sul	9	4.56 \pm 0.01	3.58	5.56	4.65	8
Santa Catarina	30	5.35 \pm 0.01	3.32	6.69	5.23	29
Sergipe	18	6.28 \pm 0.02	4.28	9.39	5.74	18
São Paulo	23	6.67 \pm 0.01	3.10	7.51	4.48	18
Mato Grosso	2	8.35 \pm 0.39	8.07	8.62	–	2
Total**	154	5.82 \pm 0.97	3.00	9.39	5.54	144

*H > 4%: Number of samples with water contents greater than 4% (w/w). ** Calculated from mean values for each region.

Table 5
Mean water contents of honeybee-collected pollen reported by national and international studies.

Origin	Reference	Botanical origin/ Processing	Method	N	Water content (%)*
Argentina	Baldi Coronel et al., 2004	Multiflower/Dehydrated	Gravimetric	37	5.8
Australia	Bell et al., 1983.	<i>Eucalyptus marginata</i> /Dehydrated	Gravimetric	1	3.2
Australia	Bell et al., 1983.	<i>Eucalyptus calophylla</i> /Dehydrated	Gravimetric	1	5.1
Cuba	Abreu, 1992.	Multiflower/NI	Gravimetric	1	10
Spain	Bonvehi and Jordá, 1997.	Multiflower/Dehydrated	Karl Fischer	20	4.9
Spain	Villanueva et al., 2002.	Commercial Multiflower/Dehydrated	Gravimetric	15	7.1
Romania	Gergen et al., 2006	Multiflower/NI	Karl Fischer	3	5.0–7.7
South of MG and SP, Brazil	Bastos et al., 2003.	Multiflower/Dehydrated (routine method of apiarists)	Karl Fischer	21	8.3
BA, MG, PR, RS, SC, SE, SP and DF, Brazil	Barreto et al., 2005.	Multiflower/Dehydrated (routine beekeeper method)	Gravimetric	42	4.0
South region, Brazil	Almeida-Muradian et al., 2005	Multiflower/Dehydrated (routine beekeeper method)	Gravimetric	10	7.4
PA, SC, RS, Brazil	Carpes, 2008.	Multiflower/Dehydrated routine beekeeper method)	Gravimetric	36	4.2

*Mean value or range; NI = not informed by author(s), N = number of samples analyzed, States of Brazil: SP = São Paulo; MG = Minas Gerais; BA = Bahia; PA = Pará; PR = Paraná; RS = Rio Grande do Sul; SC = Santa Catarina; SE = Sergipe; DF = Federal District.

majority of the samples does not comply with the Brazilian legislation currently in force, which stipulates a maximum limit of 4% for the moisture content of dried honeybee-collected pollen (BRASIL, 2001, pp. 18–23); on the other hand, most samples do comply with Argentinean food regulations, which allow a maximum value of 8%, based on FAO recommendations (Krell, 1996, pp. 87–113).

The water content of fresh pollen is variable and depends on several factors. Nonetheless, after the drying process, a product packed in an appropriate container should maintain a moisture content below 4%. Bee pollen may absorb humidity from the atmosphere if the relative humidity of the air is not appropriately controlled. In addition, the results achieved indicate that, due to the relatively wide variations in the moisture values among the different regions of origin, there is a considerable heterogeneity between pollen producers in terms of the drying techniques, methods and equipment they use. The use of driers equipped with superimposed perforated trays, temperature control and an air flow system for pollen drying, is mandatory to ensure adequate control of the water content.

Water content values greater than 4% have been observed in samples of Brazilian and international dehydrated bee pollen. In a Brazilian study, Almeida-Muradian, Pamplona, Coimbra and Barth (2005) reported mean water content values of 7.40% for 10 samples of dehydrated honeybee-collected pollen from the Southern region of the country. Barreto, Funari & Orsi (2005) studied 42 bee pollen samples from 7 Brazilian states and the Federal District, and found a mean water content value of $3.96 \pm 0.31\%$, with none of the samples coming from the states of Paraná, Rio Grande do Sul, São Paulo and the Federal District exhibiting water content levels above 4%. In a study on bee pollen conducted in Spain, Bonvehi & Jordá (1997) detected water content values varying from 4.68 to 5.87%, and water activity from 0.261 to 0.280 in 20 samples collected over two production seasons (1993 and 1994). According to these authors, the water content levels of honeybee-collected pollen determined for the purpose of their study fall within an acceptable range, ensuring adequate product stability and keeping quality during storage.

Table 5 shows mean water contents for both Brazilian and international samples of honeybee-collected pollen. It can be observed that the results reported in this study are consistent with the values of dehydrated pollen samples reported by international studies, including moisture values determined by Karl Fischer titration (Bonvehi & Jordá, 1997; Gergen et al., 2006). Since the water content values reported are high, it can be assumed that the

samples the moisture levels of which were determined had been obtained using mild drying methods. The results achieved in this study are inline with the water contents reported for Brazilian dried bee-collected pollen samples (Almeida-Muradian et al., 2005; Barreto et al., 2005), including moisture values determined by Karl Fischer titration (Bastos et al., 2003). The latter reach water content values ranging from 5.6 to 12.2% for 21 dried honeybee-collected pollen samples marketed in the states of São Paulo and Minas Gerais.

4. Conclusions

The Karl Fischer titration method can be used to determine the water contents of samples of dehydrated bee pollen with a good degree of accuracy, provided that appropriate conditions of analysis are assured, such as the particle size, sample weight, testing temperature and the titration solvent. In addition, the results obtained by Karl Fischer titration are comparable to those achieved by the traditional gravimetric method.

The results obtained for the Brazilian honeybee-collected pollen samples show that it is difficult for beekeepers to keep the water content of dehydrated bee pollen below 4%, as recommended by the Brazilian legislation. The water content values observed in this paper are consistent with those reported in national and international studies.

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References

- Abreu, M. (1992). El pollen como alimento en la nutrición humana. *Alimentaria*, 235, 45–46.
- Almeida-Muradian, L. B., Pamplona, L. C., Coimbra, S., & Barth, O. M. (2005). Chemical composition and botanical evaluation of dried bee pollen pellets. *Journal of Food Composition and Analysis*, 18, 105–111.
- Baldi Coronel, B., Grasso, D., Pereira, S. C., & Fernández, G. (2004). Caracterización bromatológica del pólen apícola argentino. *Ciencia, Docencia y Tecnología*, 29, 145–181.
- Barreto, L. M. R. C., Funari, S. R. C., & Orsi, R. de O. (2005). Composição e qualidade do pólen apícola proveniente de sete estados brasileiros e do distrito federal. *Boletim de Indústria Animal*, 62, 167–175.
- Bastos, D. H. M., Rocha, C. L., Cunha, I. B. da S., Carvalho, P. de O., & Torres, E. A. S. (2003). Composição e qualidade de pólen apícola comercializado em algumas

- idades nos estados de São Paulo e Minas Gerais – Brasil. *Revista do Instituto Adolfo Lutz*, 62, 239–244.
- Bell, R. R., Thornber, E. J., Seet, J. L. L., Groves, M. T., Ho, N. P., & Bell, D. T. (1983). Composition and protein quality of honeybee-collected pollen of *Eucalyptus marginata* and *Eucalyptus calophylla*. *Journal of Nutrition*, 113, 2479–2484.
- Bonvehí, J. S., & Casanova, T. M. (1987). Estudio analítico para determinar la humedad del pólen. *Anales de Bromatología*, 39, 339–349.
- Bonvehí, J. S., & Jordà, R. E. (1997). Nutrient composition and microbiological quality of honeybee-collected pollen in Spain. *Journal Agricultural Food Chemistry*, 45, 725–732.
- Brasil – Ministério da Agricultura, Pecuária e do Abastecimento – Instrução Normativa no 3, de 19 de janeiro de. (2001). *Regulamento técnico para fixação de qualidade e identidade de pólen apícola*. Diário Oficial da União, 23/01/01, Seção I, p. 18–23.
- Campos, M. G. R., Bogdanov, S., Almeida-Muradian, L. B., Szczesna, T., Mancebo, Y., Christian, F., et al. (2008). Pollen composition and standardization of analytical methods. *Journal of Apicultural Research*, 47, 154–161.
- Carpes, S. T. (2008). *Estudo das características físico-químicas e biológicas do pólen apícola de Apis mellifera L. da Região Sul do Brasil. [tese]*. Curitiba (PR): Universidade Federal do Paraná.
- Gergen, I., Radu, F., Bordean, D., & Isengard, H.-D. (2006). Determination of water content in bee's pollen samples by Karl Fischer titration. *Food Control*, 17, 176–179.
- Heinze, P., & Isengard, H.-D. (2001). Determination of water content in different sugar syrups by halogen drying. *Food Control*, 12, 483–486.
- Horwitz, W. (Ed.). (2005). *Official methods of analysis of the Association of Official Analytical Chemists* (18th ed.). Gaithersburg, Maryland: AOAC. (Chapter 44, method 969.38, p. 26).
- Isengard, H.-D., Schultheiß, D., Radovic, B., & Anklam, E. (2001). Alternatives of official analytical methods used for the water determination in honey. *Food Control*, 12, 459–466.
- Isengard, H.-D. (2001). Water content, one of the most important properties of food. *Food Control*, 12, 395–400.
- Jodral, M., Fernández, C., Bentabol, A., & Liñán, E. (1992). El pólen apícola com substrato para la producción de aflatoxinas. *Alimentaria*, 236, 67–69.
- Krell, R. (1996). *Value-added products from beekeeping*. In *FAO Agricultural Services Bulletin*, Vol. 124. Rome: Food and Agriculture Organization, 87–113.
- Linskens, H. F., & Jorde, W. (1997). Pollen as food and medicine – A review. *Economic Botany*, 51, 78–87.
- Loper, G. M., Standifer, L. N., Thompson, M. J., & Gilliam, M. (1980). Biochemistry and microbiology of bee collected almond (*Prunus dulcis*) pollen and bee bread. I. Fatty acids, vitamins and minerals. *Apidologie*, 11, 63–73.
- Louveaux, J. (1988). In Apimondia. (Ed.), *Bees and their breeding* (pp. 207–229), Bucharest.
- Masson, B. (1994). *O pólen*. São Paulo: Gaia, pp. 103.
- Morgano, M. A., Faria, C. G., Ferrão, M. F., Bragagnolo, N., & Ferreira, M. M. C. (2008). Determinação de umidade em café cru usando espectroscopia NIR e regressão multivariada. *Ciência e Tecnologia de Alimentos*, 28, 12–17.
- Vieira, S. (1981). Teste t. Análise de variância. In *Introdução à bioestatística*. Rio de Janeiro, RJ: Editora Campus. Cap. 12, pp. 125–137, cap. 13, pp. 141–155, 203 p.
- Villanueva, M. T. O., Marquina, A. D., Serrano, R. B., & Abellán, G. B. (2002). The importance of bee collected pollen in the diet: a study of its composition. *International Journal of Food Sciences & Nutrition*, 53, 217–224.