Bull. Chem. Soc. Ethiop. **2015**, 29(3), 485-490.
Printed in Ethiopia
DOI: http://dx.doi.org/10.4314/bcse.v29i3.16
ISSN 1011-3924

SHORT COMMUNICATION

DETERMINATION OF AMINO ACIDS AND PROTEIN CONTENT IN FRESH AND COMMERCIAL ROYAL JELLY FROM BULGARIA

Ralitsa Balkanska[1*] and Ivanka Zhelyazkova[2]

[1]Department of Special Branches – Bees, Institute of Animal Science, 2232 Kostinbrod, Bulgaria
[2]Department of Animal Science – Non-ruminants and other animals, Faculty of Agriculture, Trakia University, 6000 Stara Zagora, Bulgaria

(Received October 20, 2014; revised June 14, 2015)

ABSTRACT. Royal jelly (RJ) is popular among consumers around the world due to its perceived health benefits. The purpose of this study was to assess the levels of free and total amino acid profile as well as protein content in order to characterize Bulgarian RJ samples. A total of 17 fresh and commercial RJ samples from different regions of Bulgaria were analyzed. The results obtained show that proline (Pro), lysine (Lys), methionine (Met), aspartic acid (Asp), cysteine (Cys), histidine (His) were major free amino acids (FAAs) in RJ. The average content of Pro was 2.3 mg/g. The FAA content ranged from 5.5 to 6.2 mg/g of RJ. The most abundant total amino acids (TAAs) were aspartic acid (Asp), glutamic acid (Glu), lysine (Lys), leucine (Leu), serine (Ser) and proline (Pro). The average TAA content in fresh and commercial RJ were 129±10 and 114±8 mg/g, respectively. The results obtained for TAA content were used to establish a range for amino acid composition of Bulgarian RJ. The content of proteins was higher in fresh RJ than in commercial samples and this difference was significant ($p<0.05$). The following ranges were observed for fresh and commercial samples 14.7–17.3 and 12.5–14.9 mg/g, respectively.

KEY WORDS: Royal jelly (RJ), Free amino acids (FAAs), Total amino acids (TAAs), Protein content, Bulgaria

INTRODUCTION

Royal jelly (RJ) is a secretion produced in the hypopharyngeal and mandibular glands, located in the heads of young worker honey bees *Apis mellifera* L. [1, 2]. It is fed to all bee larvae for three days and to queen larvae for all her life, and it plays a key role in honey bee caste determination [3-5].

The chemical composition of RJ has been studied by several authors, but data available in the literature is highly variable due to the intrinsic variability of the product and the use of different analytical methods, since no reference methods have been established so far for RJ [6]. RJ contains water, proteins, sugars, lipids, amino acids, vitamins and mineral elements [7-10]. More recently sugar composition, water content, protein and lipid contents tend to be accepted for the most common criteria used for characterizing RJ quality [6]. Among these criteria, the amino acids may also provide useful information on quality based on the presence of free amino acids (FAAs) and total amino acids (TAAs) in RJ.

A lot of factors may affect the protein and amino acid fractions and they should play an important role in assessing the commercial quality of RJ during the shelf life of the product. For example, the browning reaction in RJ stored at room temperature in contact with air is appreciable after a few weeks [11].

According to Boselli *et al.* [12] the total FAA content was 7.3 mg/g RJ on average; the major FAAs were Pro, Lys, β-Ala, Phe, Ser, aspartate and glutamate. The FAA content was constant throughout storage at 4 ^{o}C. However, at room temperature, Pro and Lys increased after three months. FAAs are involved in the browning process that occurs during the commercial

*Corresponding author. E-mail: r.balkanska@gmail.com

storage of food. Our review of the literature showed that there are few papers related to the determination of amino acids in RJ samples [13-15] and one article is based on determination of FAAs in dietary supplements containing RJ [16]. According to Liming *et al*. [15] the average contents of FAA and TAA in fresh RJ were 9.21 mg/g and 111 mg/g, respectively; the major FAAs were Pro, Gln, Lys, Glu, and the most abundant TAAs were Asp, Glu, Lys and Leu.

The proteins, sugars and 10-hydroxy-2-decenoic acid (10-HDA) are the most common criteria used for RJ quality analysis and authenticity. Also amino acids could be good indicators of the origin of RJ. In this respect, the amino acid composition has not been extensively investigated for Bulgarian RJ samples in spite of the important role of these components in the quality of RJ. Therefore, determination of the content of TAAs and FAAs may be an effective way for accessing the freshness of RJ. The aim of this work was to quantitatively determine free and total amino acids in fresh and commercial RJ samples from Bulgaria.

EXPERIMENTAL

Instrumentation. Gas chromatographic method with flame ionization detector (GC-FID) was applied for identification and determination of FAAs in RJ samples. Chromatographic conditions: GC-FID chromatograms were measured on a Thermo Scientific GC-FID device, supplied with: Fast analytical column (10 m x 0.25 mm), injector temperature (split-splittless in split regime) 250 ^{o}C, temperature of flame-ionization detector 320 ^{o}C, the carrier gas flow rate 2.0 mL/min.

Total amino acid analysis was done on acid hydrolysates of RJ samples by the ion-exchange chromatography technique using an Automatic Amino Acid Analyzer (T 339 M Mikrotecha Praha). The amino acids methionine (Met) and cystine (Cys) were determined after oxidation by method of Moore and Stein [17].

Reagents and samples. All reagents were analytical grade. Reference certified standard solution with 200 nmol/L of standard substance of amino acids and norvaline solution (internal standard) were used (Sigma, 99% purity). The individual amino acid standards were purchased from Sigma. A total of 17 fresh and commercial RJ samples from different regions of Bulgaria were analyzed. All samples were kept at -18 ^{o}C before analysis. The distributors also keep commercial RJ samples at -18 ^{o}C.

Extraction of FAAs. RJ (100 mg) was dissolved with 1.0 mL 0.1 M HCl (Reagecon) and dispersed for 2 hours with the help of the sonicator and homogenized for 2 min. Samples were neutralized by adding of Na_2CO_3 (Fisher Chemical) to pH: 2.5–4.0. To 100 µL of the neutralized hydrolyzed sample, 100 µL solution of internal standard Norvaline (Sigma) were added. The obtained solutions were purified by cation-exchange solid-phase extraction. The amino acids in purified sample were derivatized with ethylchloroformate (Sigma). The derivatizing reagent was removed by scavenge with nitrogen. The derivatives of amino acids were dissolved in aliquot part of isooctane and were analyzed by GC-FID.

Protein content. Protein concentration was determined by the method of Lowry by using Folin-Ciocalteu reagent (Merck) [18]. Briefly, the Lowry method was carried out using 0.1 g RJ dissolved in 100 mL bidistilled water. Folin-Ciocalteu reagent was added and the absorbance measured at 750 nm. The standard curve where constructed using BSA (bovine serum albumin, Sigma).

Statistical analysis. The data of amino acids and protein content for all analyzed fresh and commercial RJ were compared statistically using Independent Samples T Test (SPSS Statistical Package, version 21 for Windows). Level of statistical significance was defined as $p<0.05$.

RESULTS AND DISCUSSION

Although the composition of freshly harvested and commercial RJ has been studied extensively [19, 20], the content of amino acids in RJ have received little research attention. In the present study, the results for FAAs in fresh RJ samples are presented in Table 1. The most abundant FAA in RJ was Pro (average 2.3 mg/g). Crailsheim and Leonhard [21] and Leonhard and Crailsheim [22] determined high concentration of free Pro in bee haemolymph. It should be supposed that free Pro in RJ is transferred from the workers haemolymph. Furthermore, it can be observed that Pro is not an essential amino acid. According to Micheu *et al.* [23] Pro is involved in flight metabolism of the honey bees. The amino acids Gly, Leu, Asn, Gln are under limit of detection (LOD), (under 0.009 mg/g). The concentration of Ala, Ile, Thr, Phe, Tyr is between the limit of detection and 0.1 mg/g. The limit of detection (LOD) is under 0.009 mg/g. The relative standard deviation (RSD %) is from 1 to 3%.

Table 1. FAAs in fresh RJ samples (mg/g), (n = 6).

Amino acid	Average
Valine (Val)	0.2
Serine (Ser)	0.2
Proline (Pro)	2.3
Aspartic acid (Asp)	0.3
Methionine (Met)	0.4
Glutamic acid (Glu)	0.2
Cysteine (Cys)	0.2
Lysine (Lys)	1.9
Histidine (His)	0.2
Tryptophan (Trp)	0.2

The second major integrant was Lys (1.9 mg/g) followed by Met (0.4 mg/g) Asp (0.3 mg/g), Cys (0.2 mg/g), His (0.2 mg/g). The analyzed RJ samples were not rich in FAAs. The FAA content ranged from 5.5 to 6.2 mg/g of RJ. The relative standard deviation (RSD %) of the amino acids was acceptable for the FAAs and ranged from 1 to 3%.

As has been reported by Liming *et al.* [15], the average content of FAA in fresh RJ was 9.2 mg/g and Pro was the major amino acid with an average concentration of 3.2–3.5 mg/g. The same authors reported that the major FAAs were Pro, Lys, Glu, Gln. Our results show that these FAAs are Pro, Lys, Met, Asp, Cys, His. According to Boselli *et al.* [12] the FAA content ranged from 5 to 15 mg per g of RJ. The average content of Pro and Lys is 4.0 and 1.4 mg/g, respectively. These results are similar to the results in the present study.

In a second step, the content of TAAs was determined in fresh and commercial RJ samples, using a method of ion-exchange chromatography in an automatic amino acid analyzer. The results for average values and standard deviations are listed in Table 2. The aspartic acid (Asp) has the highest concentration (24.6±1.8 and 22.4±1.5 mg/g) of all amino acid detected in fresh and commercial RJ. There was no significant difference between the content of Asp for both group of samples. The next most abundant amino acids were Glu, Lys and Leu. The amino acid Met has the lowest concentration of all amino acids 2.0±0.5 in fresh and 1.2±0.2 mg/g in commercial samples. The average TAA content in fresh and commercial RJ was 129±10 mg/g and 114 ±8 mg/g, respectively. These data were also in agreement with previous literature [15].

Significant differences between fresh and commercial RJ samples were found for the most studied amino acids (Table 2). Indeed, bee pollen and honey are precursors of RJ. Honey bees forage different plants. Thus, bee pollen and honey are always a mixture of several sources. The amino acids profile of bee pollen and honey may influence the amino acids composition of RJ in some directions. It should be mentioned that all Bulgarian RJ samples were harvested during the

flowering period of different plants from May to September. It was also found that in same causes RJ composition, mainly proteins, depends on storage conditions of the product [11]. As expected, sometimes RJ cannot be sold immediately after harvesting. For this reason, it is very important RJ to be stored in appropriate conditions. On the other hand, many authors [12, 13, 15] have reported that the content of amino acids had no significant difference throughout storage at low temperature (-18 ^{o}C). However, Maillard reaction between proteins (amino acids) and sugars is responsible for degradation processes in different products under high temperature. Other reasons for the differences between amino acids content in RJ samples are collecting condition and ages of the worker larvae [14].

Table 2. Content of TAAs in fresh (n = 12) and commercial samples (n = 5), (mg/g).

Fresh samples		Commercial samples	
Amino acids	$\bar{x}$±SD	$\bar{x}$±SD	Significance
Aspartic acid (Asp)	24.6±1.8	22.4±1.5	NS
Threonine (Thr)	6.4±0.5	5.8±0.5	p<0.05
Serine (Ser)	8.6±0.7	7.7±0.6	p<0.05
Glutamic acid (Glu)	14.5±1.3	12.9±1.2	p<0.05
Proline (Pro)	7.5±1.3	5.8±0.6	p<0.05
Glycine (Gly)	4.6±0.3	4.2±0.3	NS
Alanine (Ala)	4.3±0.3	3.7±0.3	p<0.05
Valine (Val)	6.5±0.4	6.0±0.5	NS
Methionine (Met)	2.0±0.5	1.2±0.2	p<0.05
Isoleucine (Ile)	5.1±0.3	4.6±0.4	p<0.05
Leucine (Leu)	9.6±0.6	8.7±0.7	NS
Tyrosine (Tyr)	6.5±0.4	6.0±0.5	p<0.05
Phenylalanine (Phe)	5.6±0.4	5.2±0.4	NS
Histidine (His)	4.3±0.3	3.6±0.2	p<0.05
Lysine (Lys)	10.6±0.8	9.4±0.5	p<0.05
Arginine (Arg)	6.6±0.7	6.0±0.5	NS
Total	129±10	114±8	

NS – not significant; data expressed as mean±SD.

In addition, the results showed that TAAs such as Asp, Gly, Val, Leu, Phe and Arg had no significant differences in fresh and commercial RJ samples (Table 2). Parameters such as sugars, water content, protein and 10-hydroxy-2-decenoic acid contents are the most common criteria used to characterize RJ quality. Amino acids which do not have significant differences in fresh and commercial RJ sample could be use as good indicators of freshness of RJ. Hence, they are connected with the quality of RJ. These results have to be supplemented with the results for FAAs in order to confirm and to complete these findings.

Minimal and maximal concentrations of TAAs found in all analyzed RJ samples are reported in Table 3. As can be seen from the table the minimal and maximal values do not vary in large ranges. As it was mentioned above in general, the small differences between the results in this study and in the other report are considered to be related by the influence of various factors.

Finally, the protein content of fresh and commercial RJ samples was determined. It was also observed that the protein content was significant higher in fresh samples ($p<0.05$). As can be expected this correspond with the higher content of total amino acids in the fresh samples. The following ranges were observed for fresh and commercial samples 14.7–17.3 and 12.5–14.9 mg/g, respectively. The mean values were 16.1±0.8 and 13.2±1.0%. According to Pavel *et al.* [15] there is no difference in protein content in fresh and commercial RJ samples (average 13.0±1.8%). This result is similar to our results for commercial samples. Barnutiu *et al.* [24] reported 14.7±0.3% for protein content in fresh RJ.

Table 3. Minimal and maximal values of TAAs for all analyzed Bulgarian RJ samples (n = 17), (mg/g).

Amino acids	Ranges	Amino acids	Ranges
Aspartic acid (Asp)	21.2–24.9	Isoleucine (Ile)	4.3–5.3
Threonine (Thr)	5.5–6.7	Leucine (Leu)	8.3–10.0
Serine (Ser)	7.3–8.9	Tyrosine (Tyr)	5.7–6.89
Glutamic acid (Glu)	12.1–15.0	Phenylalanine (Phe)	5.0–5.9
Proline (Pro)	5.4–7.5	Histidine (His)	3.4–4.3
Glycine (Gly)	4.0–4.8	Lysine (Lys)	9.0–10.6
Alanine (Ala)	3.6–4.3	Arginine (Arg)	5.5–6.8
Valine (Val)	5.6–6.9	Total	110–130
Methionine (Met)	1.0–2.0		

In our study, a higher protein content, as found in the fresh RJ samples compared to the commercial ones, can not indicate a higher RJ quality. Therefore, the lower protein content in commercial RJ might be due to high variance of the individual samples. It should be kept in mind that there are variable factors that could influence the protein content such as larval age of grafting of the samples or prolonged storage time of the product at the market. Figure 1 shows the protein content in fresh and commercial RJ samples. Sample 13 has a value 14.9% is a outlier.

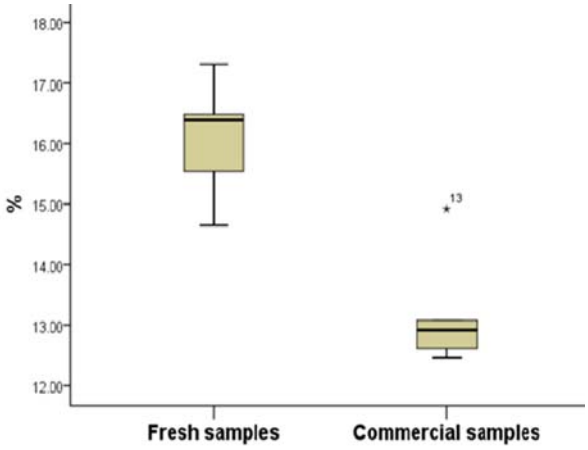

Figure 1. Box plot of protein content in fresh and commercial RJ samples. For each group of sample, mean, standard deviation, maximum, and minimum values are shown.

CONCLUSION

This study can be considered as a first approach to characterize Bulgarian RJ based on its amino acid content. Free and total amino acids were analyzed in fresh and commercial RJ samples. The results showed that RJ contained free amino acids at characteristic low levels. Proline is the major free amino acid. Furthermore, the content of proline may provide useful information when used in combination with other parameters for determination of quality of RJ. It is noteworthy that fresh RJ contained higher average values of proteins ($p<0.05$).

REFERENCES

1. Munstedt, K.; Von Georgi, R. *Am. Bee J*. **2003**, 143, 647.
2. Cruz, G.C.N.; Garcia, L.; Silva, A.J.; Barbosa, J.A.R.G; Ricart, C.A.O.; Freitas, S.M.; Sousa, M.V. *Apidologie* **2011**, 42, 252.
3. Isidorov, V.A.; Bakie, S.; Grzech, I. *J. Chromatogr. B* **2012**, 885–886, 109.
4. Chen, J.; Li, J. *Agric. Sci. China* **2009**, 8, 1219.
5. Daniele, G.; Casabianca, H. *Food Chem*. **2012**, 134, 1025.
6. Wytrychowski, M.; Chenavas, S.; Daniele, G.; Casabianca, H.; Batteau, M.; Guibert, S.; Brion, *B. J. Food Comp. Anal.* **2013**, 29, 126.
7. Isidorov, V.A.; Czyzewska, U.; Isidorova, A.G.; Bakier, S. *J. Chromatogr. B* **2009**, 877, 3776.
8. Simuth, J.; Bilikova, K.; Kovacova, E.; Kuzmova, Z.; Schroder, W. *J. Agric. Food Chem*. **2004**, 52, 2154.
9. Koya-Miyata, S.; Okamoto, I.; Ushio, S.; Iwaki, K.; Ikeda, M.; Kurimoto, M. *Biosci. Biotechnol. Biochem*. **2004**, 68, 767.
10. Scarselli, R.; Donadio, E.; Giuffrida, M.G.; Fortunato, D.; Conti, A.; Balestreri, E.; Felicioli, R.; Pinzauti, M.; Sabatini, A.G.; Felicioli, A. *Proteomics* **2005**, 5, 769.
11. Chen, C.; Chen, S.Y. *Food Chem*. **1995**, 54, 195.
12. Boselli, E.; Caboni, M.F.; Sabatini, A.G.; Marcazzan, G.L.; Lercker, G. *Apidologie* **2003**, 34, 129.
13. El-Berry, A.A.; Abdel-Gawaad, A.A.; Khattab, M.M. *Proceedings of the Fourth International Conference on Apiculture in Tropical Climates*, Cairo, Egypt **1989**, p 53.
14. Serra Bonvehi, J. *An. Bromatol*. **1990**, 62, 353.
15. Liming, W.; Jinhui, Z.; Xiaofeng, X.; Yi, L.; Jing, Z. *J. Food Comp. Anal*. **2009**, 22, 242.
16. Akamatsu, S.; Mitsuhashi, T. *J. Food Comp. Anal.* **2013**, 30, 47.
17. Moore, S.; Stein W.H. *J. Biol. Chem*. **1954**, 211, 907.
18. Bulgarian standard, *ON 2576693-84 Fresh and Lyophilized Royal Jelly*, Ministry of Interior: Sofia; **1985**; p 1.
19. Pavel, C.; Marghitas, L.; Dezmirean, D.; Tomos, L.; Bonta, V.; Sapcaliu, A.; Buttstedt, A. *J. Apic. Res.* **2014**, 53, 116.
20. Marghitas, L.A; Morar, O.; Bobiş, O.; Bonta, V. Dezmirean, D.S. *J. Agroaliment. Proc. Technol.* **2010**, 16, 399.
21. Crailsheim, K.; Leonhard, B. *Amino Acids* **1997**, 13, 141.
22. Leonhard, B.; Crailsheim, K. *Amino Acids* **1999**, 17, 195.
23. Micheu, S.; Crailsheim, K.; Leonhard, B. *Amino Acids* **2000**, 18, 157.
24. Barnutiu, L.I.; Marghitas, L.A.; Dezmirean, D.; Bobis, O.; Bonta, V.; Pavel C. *Bull. UASVM Animal Sci. Biotechnol.* **2012**, 69, 36.