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ABSTRACT

Due to its high nutritive value, royal jelly usage is increasing, both in human nutrition in native form and as bioactive component in other products (dietary supplements, medicines). The database and regulations on royal jelly characteristics are established in several countries, but not in Croatia. Physicochemical characteristics: moisture, protein content, pH value, total acidity, carbohydrate composition and 10-HDA content in 13 Croatian royal jelly samples were determined with the aim of getting insight to quality of royal jelly produced in Croatia. The obtained results showed that regarding 10-HDA content, one of the most important quality parameter, all samples fulfilled the international standard for royal jelly specifications. Moisture of three samples was higher than prescribed (69.5%, 76.3% and 72.0%, respectively) while one sample had slightly lower protein content than minimum 11% prescribed in international standard. Sucrose content in two royal jelly samples was higher than 3%. Statistically significant correlations were obtained between moisture and protein content, 10-HDA and total acidity as well as between fructose and glucose content. The results of this study will contribute to creation the database of Croatian royal jelly physicochemical characteristics and thus help in setting the royal jelly quality criteria at national level.

Introduction

Royal jelly is one of the most appreciated honeybee's products. It is a secretion of mandibular and hypopharyngeal glands of young worker honeybees used mainly for feeding and development of queen bee. During the first three days of life, all larvae are fed with royal jelly. Afterwards only larvae destined to become queens are fed with royal jelly, while drones and worker bee larvae are fed with pollen and honey (Ferioli et al., 2007; Wytrychowski et al., 2013; Kanelis et al., 2015). The difference in feeding during larval stage is considered to be a major factor contributing to significant differences in morphology, life span and behaviour between queen and worker bees (Ferioli et al., 2007). Royal jelly is viscous, gelatinous white to bright yellow substance with sour and pungent odor, and sour and sweet taste (Bogdanov, 2016). Unlike honey, chemical composition of royal jelly is relatively constant regardless on bee breeds, different colonies and temperature variations (Bărnuţiu et al., 2012). Variations in chemical compositions are a result of different feeding (without or with sugars and/or proteins supplementation), environmental conditions, hygienic conditions of hive cells and manipulation (Isidorov et al., 2012; Kanelis et al., 2015). Fresh royal jelly contains 60 to 70% of water, up to 18% of proteins, 7 to 18% of carbohydrates, 3 to 8% of lipids and in small amount vitamins and minerals (Sabatini et al., 2009). Highly valuable nutritive composition of royal jelly is recognized by consumers and the consummation of royal jelly in different forms (native or as a functional component of many food products) is constantly growing. Studies have shown that royal jelly has beneficial properties, e.g. antibacterial, antioxidant,

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anti-inflammatory, antitumoral, antiaging and immune activating properties that are mainly attributed to the presence of (2*E*)-10-hydroxydec-2-enoic acid, 10-HDA (Oršolić, 2013; Kolayli et al., 2016, Pasupuleti et al., 2017). This unsaturated fatty acid is characteristic only for royal jelly and as such has been considered as authenticity and quality parameter of royal jelly (Sabatini et al., 2009). But, the reports on mechanism of synthetic 10-HDA production are available indicating that 10-HDA content cannot be used as exclusive authenticity parameter. With the aim of quality and authenticity assessment, besides 10-HDA content, carbohydrate and protein content, moisture, furosine content and 13C/12C isotopic ratio are usually determined (Sabatini et al., 2009; Kanelis et al., 2015; ISO, 2016; Hu et al., 2019). Like in honey, geographical origin of royal jelly can be assessed by pollen analysis (Piana et al., 2006; Dimou et al., 2007; Dimou et al., 2013). Defined and prescribed national quality criteria for royal jelly are established by few countries, while at international level the first suggestions for royal jelly specifications were reported by royal jelly working group of International Honey Commission in 2009 (Sabatini et al., 2009). In 2016, International Organization for Standardization (ISO) published international standard for royal jelly specifications that describes sensory, chemical and hygienic requirements and methods for royal jelly quality control (ISO, 2016). The Republic of Croatia has no quality standards for royal jelly, and the research conducted on Croatian royal jelly is at the beginning. Therefore, the aim of this study was to determine physicochemical characteristics of royal jelly produced in Croatia in order to start creating the database of Croatian royal jelly and thus help in setting the royal jelly quality criteria at national level.

Materials and methods

Royal jelly samples

Characterization of royal jelly was performed on 13 fresh royal jelly samples purchased from the beekeepers from eastern region of Croatia. Samples were taken from queen cells, from larvae that are 3 days old. The royal jelly samples were frozen immediately after collection in glass containers (cca 10 g) and transported to the laboratory in frozen state. The analyses were performed within the month after sample collection.

Chemicals

HPLC grade acetonitrile and methanol used of chromatographic analyses were purchased from J.T. Baker (Netherland) and phosphoric acid (for HPLC, 85 - 90%) was from Fluka, USA. Fructose (≥ 99%), glucose (≥ 99.5%), sucrose (≥ 99.5%) and methyl-4-hydroxybenzoate (≥ 99%) were purchased from Sigma-Aldrich (USA) while 10-HDA standard (≥ 99%) was purchased from Cayman Chemicals, USA. Other used chemicals were analytical grade.

Physicochemical analyses

Moisture was determined by lyophilisation method (A.3.) described in royal jelly international standard (ISO, 2016). Protein content was determined by the Kjeldahl method using a conversion factor of 6.25 and total acidity by titrimetric method. Carbohydrate composition and content and 10-HDA were determined on Shimadzu liquid chromatograph consisting of LC-20AD solvent delivery module, CTO-20AC column oven, SIL-10AF autosampler, RID-10A differential refractometric detector (for carbohydrate determination) and SPD-M20A photodiode array detector (for 10-HDA determination). Instrument was supported with LabSolution Lite software (Release 5.52). Qualitative and quantitative determination of glucose, fructose and sucrose in royal jelly was performed. Sample preparation was performed as described in International Standard 12824 (ISO, 2016). The separation of carbohydrates was performed on XBridge Amide HPLC column (Waters, USA, 4.6x150 mm, particle size 3.5 μm). Mobile phase (acetonitrile/water, 75/25 with 2% of triethylamine) flow was 1 mL/min. Injection volume was 10 μL, and the column temperature was set at 30°C. Identification of separated carbohydrates was achieved based on the retention time while the quantification was performed using the external calibration method. Fructose and glucose standard solutions used for calibration were prepared in concentration range from 0.5% to 8%, and sucrose solutions in concentration range from 0.5% to 5%. The content of fructose, glucose and sucrose in royal jelly was expressed as percentage of individual carbohydrate in fresh royal jelly (%). For 10-HDA determination royal jelly samples were prepared according to the procedure B2 in the International Standard 12824 (HPLC-UV internal standard method). Chromatographic conditions were as follows: mobile phase (methanol/water, 55/45, pH 2.5 adjusted with phosphoric acid) flow was 1 mL/min, injection volume 10 μL, column temperature was 35°C, monitoring wavelength was 190 - 400 nm, and the detection wavelength 210 nm. The chromatographic analysis was performed on HPLC column Inertsil ODS-3V (GL Sciences, 250 mm x 4.6 mm, 5 μm particle size). Identification of 10-HDA was achieved based on the retention time and comparison

of the 10-HDA absorbance spectrum of royal jelly with pure component spectrum. Internal standard used for quantification was methyl-4-hydroxybenzoate. The results were expressed as percentage of 10-HDA in fresh royal jelly (%).

Statistical analysis

All analyses were performed in duplicate. Average values and standard deviations were calculated using Microsoft Excel 2016 (Microsoft Corp.) software. The relationship between parameters was calculated using STATISTICA® 13.3 (Dell Inc., Round Rock, TX, USA) software and expressed as Pearson correlation coefficient at significance level of 0.05.

Results and discussion

In order to start creating a database of Croatian royal jelly physicochemical characteristics, 13 fresh royal jelly samples were collected from eastern Croatia. The results of moisture, protein content, pH and total acidity are presented in Table 1. Moisture content varied between 63.7% and 76.3%. The obtained results were in compliance with available literature data (Sabatini et al., 2009; Balkanska et al., 2012; Bărnuţiu et al., 2012; Wytrychowski et al., 2013; Kolayli et al., 2016; Yavuz and Gürel, 2017). The international standard (ISO, 2016) prescribed the limits of moisture in fresh royal jelly between 62.0% and 68.5% and according to these specifications three samples don't comply with the standard. Zheng et al. (2011) and Kanelis et al. (2015) reported that royal jelly moisture content is highly dependent on time of collection after the grafting of young larvae. Namely, the most often procedure for royal jelly production is to collect it three days (72 hours) after larvae is transferred into cells. Earlier collection (1 or 2 days after grafting) of royal jelly results in lower water content due to the shorter production cycle. On the other hand, latter collection (after 72 hours) also produces royal jelly with low moisture (<50%) because the bees use a part of the royal jelly's water (Kanelis et al., 2015). Some authors (Kolayli et al., 2016; Yavuz and Gürel, 2017) have also reported high moisture (>70%) in fresh royal jelly. Mărgăoan et al. (2017) compared physicochemical parameters of fresh royal jelly samples with queen bee larvae triturate and fresh Apilarnil, a product that contains drone bee larvae. The water content of queen bee larvae triturate and fresh Apilarnil was considerably higher (75.17±0.15% and 73.25±0.02%, respectively) compared to the fresh royal jelly (66.03±0.03%). The protein content of analyzed royal jelly samples was from 10.51% to 15.93%. Sample RJ10 had 10.51% of protein that is lower than prescribed in international standard (ISO, 2016) but complies with propositions of International Honey Commission working group for royal jelly (Sabatini et al., 2009). Low protein content was also reported by Kanelis et al. (2015) for Greek royal jelly (10.5 - 21.0%) and Yavuz and Gürel (2017) for royal jelly available at Turkish market. Other analyzed samples had protein content comparable to the literature data (Balkanska et al., 2012; Wytrychowski et al., 2013; Kolayli et al., 2016). Statistically significant negative correlation (r=-0.746) was obtained between moisture and protein content (Table 3). The acidity of royal jelly originates from organic acids and pH values between 3.98 and 4.21 (Table 1). Total acidity obtained in this study varied between 32.18 and 47.38 mL 1 M NaOH/100 g. This quality parameter is rather constant regardless of time of collection and geographical origin of samples. As shown in Table 3, high statistically significant positive correlation was determined between total acidity and 10-HDA content (r = 0.904). The content of 10-HDA is thought to be the most important quality and authenticity parameter considering that this unsaturated fatty acid is naturally present only in royal jelly. Fresh royal jelly samples analyzed in this study had 10-HDA content between 1.65% and 3.88% (Figure 1.). The obtained results are in accordance to our previous study (Flanjak et al., 2017) as well as to the literature data of royal jellies from different geographical origin (Wytrychowski et al., 2013; Kanelis et al., 2015; Kolayli et al, 2016; Yavuz and Gürel, 2017). International standard (ISO, 2016) suggests that the minimum content of 10-HDA in fresh royal jelly should be 1.4%, but some countries, e.g. Brazil, have established higher minimal value for 10-HDA content in authentic royal jelly. On the other hand, Kanelis et al. (2015) and Yavuz and Gürel (2017) have stressed that upper limit of 10-HDA should also be regulated due to the possible adulteration with synthetic 10-HDA. In order to set the limits of 10-HDA content in Croatian royal jellies, more samples from different regions of Croatia and several production seasons should be collected and analysed. Besides 10-HDA, the most important royal jelly authenticity parameter is carbohydrate content and composition. Royal jelly contains fructose and glucose in higher amounts and disaccharides and trisaccharides in smaller amount (Daniele and Casabianca, 2012). Among di- and trisaccharides, sucrose, maltose and erlose are most abundant. Fructose content in analyzed royal jelly samples was between 2.82% and 5.82% and glucose between 2.56 % and 6.36% (Table 2) and the high statistically significant positive correlation was determined between fructose and glucose content (r=0.855)

(Table 3). Fructose and glucose content are rather constant regardless on bee feeding with respect on addition of sugar syrups, but the significant differences occur in di- and trisaccharides content (Sesta, 2006; Daniele and Casabianca, 2012, Kanelis et al., 2015, ISO 2016). Namely, higher sucrose and erlose content in royal jelly could indicate feeding of honeybees with beet or cane sugar syrups while higher maltose and maltotriose content are found in royal jelly when honeybees are fed with cereal and corn starch syrups (Daniele and Casabianca, 2012; Wytrychowski et al., 2013). Maximal prescribed sucrose content in royal jelly for naturally fed honeybees is 3% (ISO, 2016) and according to that, two analyzed samples (RJ3 and RJ10) do not meet the recommendations (Table 2). The reason for this nonconformity is honeybees-feeding with exogenous sugar as stated by the beekeepers. Those samples had also relatively low fructose and glucose content compared to other analyzed samples. The results are in compliance with the findings of Kanelis et al. (2015) which reported that addition of sugar syrups in honeybee feeding results in lower fructose and glucose and higher sucrose content.

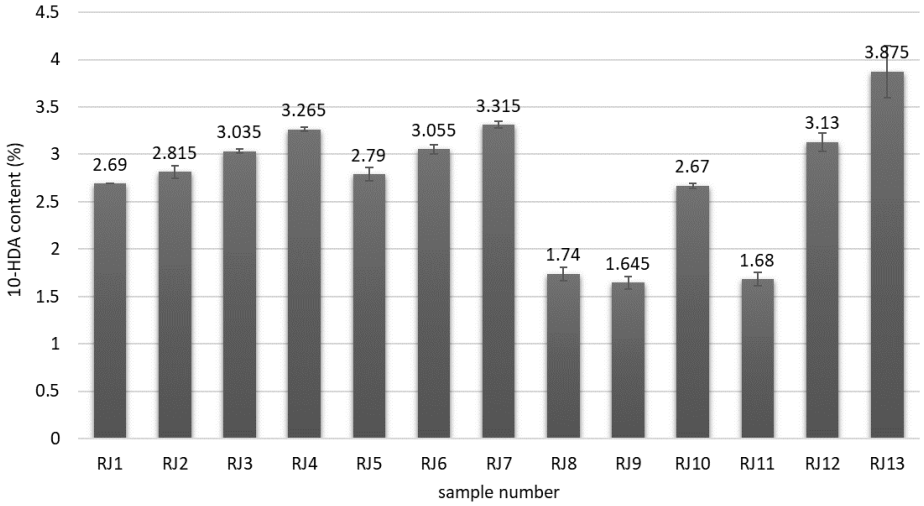

Fig 1. The average values and standard deviations of (2*E*)-10-hydroxydec-2-enoic acid (10-HDA) content in analysed fresh royal jelly samples

Table 1. Moisture, protein, pH and total acidity in analysed fresh royal jelly samples

Sample	Moisture (%)	Protein (%)	pH value	Total acidity (mL 1 M NaOH/100 g)
RJ1	63.7±0.0	15.93±0.28	4.02±0.01	45.6±0.0
RJ2	67.5±0.2	12.80±0.00	3.99±0.01	41.8±0.9
RJ3	64.3±1.9	14.36±0.00	4.02±0.01	42.7±0.6
RJ4	68.0±1.3	12.62±0.00	4.02±0.01	42.4±0.0
RJ5	69.5±0.5	12.25±0.01	4.08±0.01	39.8±0.3
RJ6	67.2±0.1	11.73±0.00	4.03±0.01	40.6±0.8
RJ7	66.4±2.4	15.28±0.02	4.21±0.00	47.3±0.7
RJ8	65.5±0.5	13.22±0.12	4.10±0.01	32.4±2.3
RJ9	66.4±0.5	13.56±0.35	4.02±0.01	32.2±0.3
RJ10	76.3±1.4	10.51±0.01	4.19±0.03	36.5±0.7
RJ11	66.8±0.2	14.18±0.24	4.03±0.01	33.4±0.3
RJ12	72.0±0.1	13.13±0.00	3.98±0.01	44.4±0.0
RJ13	66.0±0.2	13.65±0.01	3.98±0.00	47.4±0.3
Average	67.5	13.32	4.05	40.5
SD	3.5	1.44	0.08	5.4
Min	63.7	10.51	3.98	32.2
Max	76.3	15.93	4.21	47.4

Min-minimum, Max-maximum, SD-standard deviation; Values represent the average of duplicates ± standard deviation

Table 2. Fructose, glucose and sucrose content in analysed fresh royal jelly samples

Sample	Fructose (%)	Glucose (%)	Sucrose (%)
RJ1	3.59±0.00	2.56±0.05	2.80±0.02
RJ2	3.72±0.06	4.16±0.12	1.98±0.08
RJ3	3.83±0.02	3.76±0.02	4.46±0.04
RJ4	4.82±0.02	5.46±0.00	1.71±0.09
RJ5	3.13±0.07	4.53±0.08	1.86±0.01
RJ6	4.73±0.13	5.28±0.28	0.99±0.11
RJ7	3.27±0.02	4.04±0.04	0.70±0.04
RJ8	5.82±0.12	5.71±0.11	2.88±0.00
RJ9	4.94±0.19	4.94±0.19	2.12±0.19
RJ10	2.82±0.27	3.09±0.48	3.73±0.77
RJ11	5.73±0.03	6.36±0.06	1.61±0.01
RJ12	5.19±0.86	5.82±0.74	1.78±0.23
RJ13	4.16±0.01	4.51±0.01	1.28±0.00
Average	4.29	4.63	2.14
SD	0.99	1.11	1.07
Min	2.82	2.56	0.70
Max	5.82	6.36	4.46

Min-minimum, Max-maximum, SD-standard deviation; Values represent the average of duplicates ± standard deviation

Table 3. Pearsons' correlation coefficients between analysed physicochemical parameters

	Moisture	Protein	pH	Total acidity	10-HDA	Fructose	Glucose	Sucrose
Moisture	1.000							
Protein	-0.746*	1.000						
pH	0.359	-0.137	1.000					
Total acidity	-0.076	0.356	-0.131	1.000				
10-HDA	0.152	-0.020	-0.089	0.904*	1.000			
Fructose	-0.282	0.078	-0.432	-0.468	-0.474	1.000		
Glucose	-0.019	-0.168	-0.296	-0.405	-0.299	0.855*	1.000	
Sucrose	0.082	-0.074	0.072	-0.260	-0.249	-0.203	-0.451	1.000

(*) significant at $p < 0.05$; 10-HDA (2E)-10-hydroxydec-2-enoic acid

Conclusion

Increasing usage of royal jelly requires setting the quality parameters limits at national and international level. The results of this study will contribute to creation of Croatian royal jelly database but further research must include more samples from different regions of Croatia to gain the limits for each physicochemical characteristic and finally suggest the quality requirements for royal jelly produced in Croatia.

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