Changes in composition of royal jelly harvested at different times: consequences for quality standards

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Original article

Changes in composition of royal jelly harvested at different times: consequences for quality standards*

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Abstract – Most of the studies on royal jelly (RJ) composition or properties as well as quality standards of commercially available royal jelly are based on RJ harvested three days (72 h) after grafting. In China, some beekeepers produce RJ harvested one (24 h) or two (48 h) days after grafting. There is a lack of knowledge about the quality of the royal jelly harvested earlier than 72 h. This study compared 32 colonies for their chemical compositions of RJ harvested at 24, 48 and 72 h after grafting, according to the proportion of moisture, protein, 10-HDA, total sugar and the value of acidity and superoxide dismutase activity. The analysis of RJ samples revealed that the composition varied significantly (for both fresh and dehydrated samples) and on some occasions above and below the range of present Chinese and Swiss standards. The results suggest that harvesting time should be considered when defining new quality standards of RJ.

honeybee / royal jelly / chemical composition / quality standard

1. INTRODUCTION

The hypo-pharyngeal and mandibular glands of nurse honeybee workers secrete jelly which the bees feed to adults and larvae as part of their diets. This jelly plays a central role in caste determination of honeybees. Royal jelly (RJ) is fed specifically to larvae destined to become queens. The consumption of RJ by larvae affects their DNA methylation processes (Kucharski et al., 2008) and results in the development of gyne morphology. Larvae destined to become workers are fed jellies of different compositions, quantities and timings to the those fed to queens (Brouwers et al., 1987). In addition to these differences at the caste level, the composition of the jellies varies seasonally regionally and among different honeybee species. In general, jellies are composed of 60–70% moisture, 12–15% crude proteins, 3–6% lipids, 6–18% sugars (Lercker et al., 1981; Lercker et al., 1982; Howe et al., 1985).

Given its effect on caste determination in honeybees (generating 'queens': fertile individuals heading a colony), RJ has been incorporated in traditional human medicine and is widely promoted and commercially available as a medication, health food and cosmetic in many countries, especially in China and Japan. As a result, the effects of RJ consumption by humans have been studied and many physiological activities have been reported: these include hypotensive (Matsui et al., 2002), antitumor (Townsend et al., 1960), anti-inflammatory (Fujii et al., 1990), antifatigue (Kamakura et al., 2001) and antiallergic (Kataoka et al., 2001). In order to discover the compounds responsible for these beneficial effects on health, the chemical composition of RJ has been the topic of many studies. Thus, several bioactive substances have

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been identified: 10-hydroxy-2-decenoic acid, a fatty acid, which exhibits antibiotic activity against many bacteria and fungi (Blum et al., 1959), MRJP3 that modulates immune responses (Okamoto et al., 2003), a 350-kDa protein called apisin that stimulates the proliferation of human monocytes (Kimura et al., 1995), antibacterial and antioxidative peptides (Fontana et al., 2004; Guo et al., 2009).

Commercial RJ is produced by transferring (grafting) young larvae (1 day old) into artificial queen cells to induce nurse bees to provision the cell with RJ (Chen et al., 2002). The majority of RJ producers harvest three days (72 h) after grafting because at this time the amount of RJ in queen cells reaches its peak (Lercker et al., 1985). In recent years, some producers sell RJ that was harvested two days (48 h) or one day (24 h) after grafting. These earlier harvests shorten the production cycle but allow collection of similar quantities of RJ over the same period as the traditional method. Since RJ is stored at hive temperature (35 degrees Celsius) and humidity (60–80% RH) for one or two days less than the common RJ (harvested 72 h post-grafting) the producers argue that the "Early Harvest" RJ is fresher. However, since most of the studies on RJ composition or properties as well as the quality standards of commercially available RJ are based on it being harvested 72 h after grafting, there is a lack of knowledge about the quality of RJ harvested earlier than 72 h.

Only few studies have been conducted to elucidate the changes in composition of larval food over time. Some of the studies aimed at identifying the chemical determinants of caste differentiation during larval development (Mitsui et al., 1964; Brouwers, 1984; Brouwers et al., 1987). Shen (1991) and Chen et al. (1992) also compared the composition of RJ harvested 48 h and 72 h after grafting in order to identify freshness indicators. Despite these few studies, existing quality standards do not cover the emerging range of RJ's.

Since chemical characterisation is necessary for quality control of internationally marketed natural products for human consumption, here we attempt to fill the gap left by the Early Harvest RJ's. We did this by analysing the composition of the RJ harvested at 24, 48 and 72 h after grafting in more detail and with higher sample size than previous studies. China is the world's largest RJ producer. Thus, our aim was to evaluate differences in quality according to RJ production methods used in China. We analysed moisture, protein, sugar, 10-HDA and acidity which are the most common criteria used to determine RJ properties (Sabatini et al., 2009) and compared the compositions of Early Harvest RJ with the established quality standards of China and Switzerland. In order to investigate the freshness of Early Harvest RJ, we also analysed superoxide dismutase (SOD) activity. Depending on how rapidly their quantity change with time, only some of the constituents of RJ represent good freshness markers within the first three days of its production. We used SOD because it was found to be highly sensitive to storage temperatures, its activity changes rapidly over 10 days (Zhang et al., 1996; Tang and Yuan, 1999), and has been purified from RJ (Min et al., 2004). Therefore it could be an ideal marker for assessing the freshness of Early Harvest RJ.

Our results could contribute to the establishment of new quality standards for RJ products and to the evaluation of their freshness.

2. MATERIALS AND METHODS

2.1. Samples

At each of four apiaries in Tonglu county, Zhejiang province, China, 8 colonies of *Apis mellifera ligustica* were randomly chosen to produce fresh royal jelly. To avoid any possible effect of kin recognition on nursing behaviour of the queen larvae (Moritz et al., 2005), a colony which did not have genetic relationship with the nursing colonies was used to provide young larvae in each apiary. Worker larvae of approximately 1 day old were transferred from their cell into plastic queen cell cups (Zhejiang Donghai Apicultural Company, DU-II Type). One frame containing four strips of plastic queen cups (n = 33 per strip), was used in each colony. This frame was placed into the honey compartment of the hives. Cups were cleaned to ensure that no residual RJ remained and reused for several production cycles. At 24 h, 48 h and 72 h after grafting, the frames of queen cells were removed from the colonies. The

wax that workers built over the cups to form the cells and larvae in the cells were removed. The RJ contained in the cup was collected with a microspatula and then pooled with the rest of the RJ obtained from the same hive (33 × 4 cups) on each occasion. To minimize the effect of water evaporation during collection, RJ was collected within 3 min of queen cells removal from the colonies. It was then immediately frozen at –20 °C and conserved at this temperature until chemical analysis was performed. This temperature was shown to provide optimal conditions for the conservation of RJ (Chen and Chen, 1995). The RJ sampling occurred in April 2006, when honeybees foraged on rape, which was their major floral source available at this time.

Royal jellies produced 24 h, 48 h and 72 h after grafting are termed 24 h RJ, 48 h RJ and 72 h RJ respectively, hereafter.

2.2. Chemical analyses

The analysis of moisture, crude protein, total sugar, acidity, 10-HDA followed the methods of the quality standard of royal jelly published in 2002 by the General Administration of Quality Supervision, Inspection and Quarantine of the People's Republic of China (GB/T 9697–2002) (General Administation of Quality Supervision, Inspection and Quarantine of the People's Republic of China, 2002). Moisture was measured by weight loss upon drying in a vacuum drying oven. The quantity of crude protein was calculated by multiplying the content of nitrogen, determined by Kjeldahl's method, by 6.25. The total amount of sugar was determined by the Fehling method after hydrolyzing the sample in HCl (6 mol/L) for 10 min and calculating the glucose equivalent. Acidity was determined by titration with NaOH and presented in terms of neutralization by mL/1N NaOH/100g sample. 10-HDA was determined by high-performance liquid chromatography (HPLC) (Bloodworth et al., 1995).

For SOD quantification, 1 g of RJ was homogenised in 5 mL Tris-HCl buffer (50 mmol/L, pH 8.2) and the supernatant was used after being centrifuged at 6000 r/min for 20 min. SOD activity was determined by the pyrogallol auto-oxidation method (Zou et al., 1986). One unit of SOD activity was defined as 50% inhibition of the uninhibited rate of pyrogallol autooxidation. Activity data was expressed as units of SOD activity per gram of RJ.

In order to assess the composition of RJ without the diluting effect of its water content and compare our values with studies that measured lyophilized RJ, we calculated a dry basis weight using the following formula:

$$X \times 100/(100 - W)$$

where 'X' represents the measured wet weight of a component of RJ and 'W' the water content of this sample.

2.3. Statistical analyses

The data obtained from the various apiaries was first compared using ANOVA to determine whether it represented samples form a unique population and whether it could be pooled for further analyses. The data was normally distributed and of homogeneous variance for all variables with the exception of acidity of 24 h RJ. A Kruskall-Wallis test was performed to verify whether acidity of 24 h RJ varied between apiary and was found to be similar in the four apiaries (Kruskal-Wallis Test Statistic = 5.5, df = 3, $P = 0.14$). The jelly harvested from all apiaries was found to have similar composition, acidity and SOD activity, with the following exceptions: moisture of 24 h RJ and SOD activity of 48 h RJ. Given that the other variables or these variables on other days did not show variations between apiaries, we pooled all the data.

Acidity, moisture, SOD activity and quantity of 10-HDA, protein and sugar were compared between 24 h RJ, 48 h RJ and 72 h RJ using ANOVA. Data were log transformed to normalize their distribution when necessary or arcsine transformed when expressed as percentage to prevent dependence of the variance on the mean. In the case of 10-HDA on wet basis, data was not normally distributed and transformation failed to normalise them, thus non-parametric statistics (Mann-Whitney U-test) were used instead. Post-hoc comparisons were performed with Tukey's Honestly-Significance-Difference test following the ANOVA analysis and with paired comparisons with Mann-Whitney U-test after non parametric statistics were used. In cases where the requirement of homogeneity of variance between groups was not met for the ANOVA analysis, post-hoc comparisons were done with the Games-Howell test (for water and acidity on both wet and dry basis). For the cases in which the same set of data was used several times in the comparisons with Mann-Whitney U-test, a Bonferroni correction was applied.

Table I. Composition of royal jelly harvested 24, 48 and 72 h after grafting (wet basis, n = 32 colonies, $\bar{x} \pm$ SD).

Samples	24 h RJ	48 h RJ	72 h RJ
moisture (%)	53.3 ± 4.7	62.8 ± 2.1	64.3 ± 1.8
crude protein (%)	19.6 ± 1.4	16.2 ± 1.5	15.0 ± 1.0
sugar (%)	14.3 ± 1.8	12.4 ± 1.5	12.1 ± 1.4
acidity (mL 1N NaOH/100g)	47.3 ± 4.1	39.4 ± 3.2	37.2 ± 3.0
10-HDA (%)	2.5 ± 0.4	2.0 ± 0.3	2.1 ± 0.2
SOD (U/g)	134.0 ± 48.1	147.0 ± 51.1	139.8 ± 28.1

Table II. Composition of royal jelly harvested 24, 48 and 72 h after grafting (dry basis, ANOVA, n = 32 colonies, $\bar{x} \pm$ SD). The single significant differences (ANOVA) occur for SOD activity of 24 h RJ, which is significantly lower than that of 48 h RJ and 72 h RJ.

Samples	24 h RJ	48 h RJ	72 h RJ
Crude protein (%)	42.2 ± 4.4	43.7 ± 4.1	42.1 ± 2.7
Sugar (%)	30.6 ± 3.3	33.2 ± 3.4	33.9 ± 3.71
Acidity	102.2±12.7	106.1±9.2	104.0±9.1
10-HDA (%)	5.5 ± 0.8	5.5 ± 0.9	5.9 ± 0.8
SOD (U/g)	291.7 ± 118.6	392.1 ± 124.8	392.2 ± 78.0

In addition, multivariate statistics discriminant analyses were used to compare the composition, acidity and SOD activity profile of the RJ harvested on different days on wet and dry basis. Samples with missing values for one or more RJ components were removed from these analyses. The relative amounts of the compounds were restandardised to 100% and transformed using Aitchison's formula (1986):

$$Z_{ij} = \ln(Y_{ij}/g(Y_j))$$

where Z_{ij} is the standardized peak area I for individual j, Y_{ij} is the peak area I for individual j and $g(Y_j)$ is the geometric mean of all peaks for individual j.

Statistical analyses were performed with Systat 12.02.00.

3. RESULTS

Table I shows the chemical composition of 24 h RJ, 48 h RJ, 72 h RJ. Moisture in RJ increased with time and each harvesting day showed a significant difference compared to the others (ANOVA df = 2: F = 115.2, $P < 0.01$; Tukey HSD test $p_{24-48\ h\ RJ} < 0.01$, $p_{24-72\ h\ RJ} < 0.01$; $p_{48-72\ h\ RJ} < 0.01$). However, the difference in moisture was greater between 24 h RJ and 48 h RJ than between 48 h RJ and 72 h RJ. The proportion of 10-HDA decreased with time and was significantly higher in 24 h RJ. 48 h RJ and 72 h RJ had similar proportions of 10-HDA (Mann-Whitney U-test, df = 1, $U_{24-48\ h\ RJ} = 868.0$, $p_{24-48\ h\ RJ} < 0.01$; $U_{24-72\ h\ RJ} = 856.0$, $p_{24-72\ h\ RJ} < 0.01$; $U_{48-72\ h\ RJ} = 401.0$, $p_{48-72\ h\ RJ} = 0.14$). The proportion of sugar followed the same pattern (ANOVA df = 2: F = 17.7, $P < 0.01$; Tukey HSD test, $p_{24-48\ h\ RJ} < 0.01$; $p_{24-72\ h\ RJ} < 0.01$; $p_{48-72\ h\ RJ} = 0.73$), as did acidity (ANOVA df = 2: F = 68.8, $P < 0.01$; Tukey HSD test, $p_{24-48\ h\ RJ} < 0.01$; $p_{24-72\ h\ RJ} < 0.01$; $p_{48-72\ h\ RJ} = 0.02$). The proportion of proteins decreased significantly on consecutive days (ANOVA df = 2: F = 102.5, $P < 0.01$; Tukey HSD test, $p_{24-48\ h\ RJ} < 0.01$; $p_{24-72\ h\ RJ} < 0.01$; $p_{48-72\ h\ RJ} < 0.01$). SOD activity was similar for RJ harvested on consecutive days (ANOVA df = 2, F = 0.72, $P = 0.49$; Tukey HSD test, $p_{24-48\ h\ RJ} = 0.46$; $p_{24-72\ h\ RJ} = 0.86$; $p_{48-72\ h\ RJ} = 0.76$).

When considering the amount of components on dry basis (Tab. II), we found no significant differences among the variables (df = 2, $F_{10-HAD} = 2.8$, $p_{10-HAD} = 0.08$; $F_{protein} = 1.6$, $p_{protein} = 0.21$; $F_{acidity} = 1.1$, $p_{acidity} =$

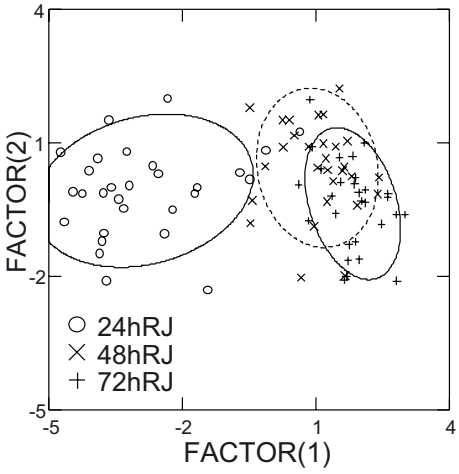

Figure 1. Discriminant analysis of the fresh RJ sampled 24, 48 and 72 h after grafting.

0.36) except for the SOD activity of 24 h RJ, which was significantly lower than that of 48 h RJ and 72 h RJ (ANOVA, df = 2, F = 9.0, $P < 0.01$, Tukey HSD test $p_{24-48\ h\ RJ} < 0.01$, $p_{24-72\ h\ RJ} < 0.01$, $p_{48-72\ h\ RJ} = 1.0$) and for sugar dry weight (ANOVA df = 2, F = 7.9, $P < 0.01$, Tukey HSD test $p_{24-48\ h\ RJ} < 0.01$, $p_{24-72\ h\ RJ} < 0.01$, $p_{48-72\ h\ RJ} < 0.75$) which also showed the same trend.

The discriminant analysis (Lambda = 0.17, df = (6, 2, 84), F-ratio = 19.04, df = (12, 158), $P < 0.01$), showed that the properties and composition of fresh 48 h RJ and 72 h RJ were more similar to each other compared to fresh 24 h RJ (Fig. 1, between group F-matrix $F_{24-48\ h\ RJ} = 35.20$; $F_{24-72\ h\ RJ} = 50.34$; $F_{48-72\ RJ} = 2.90$). The results for the discriminant analysis performed on the composition of dehydrated RJ are similar (Lambda = 0.64, df = (4,2,84), F-ratio = 5.12, df = (8,162), $P < 0.01$; between group F-matrix $F_{24-48\ h\ RJ} = 6.65$; $F_{24-72\ h\ RJ} = 6.79$; $F_{48-72\ h\ RJ} = 2.42$). However, the difference between 24 h RJ and RJ harvested later appears greater when moisture is considered, resulting in a poorer discrimination between the age groups in dehydrated samples (Tab. III).

4. DISCUSSION

Our results show that the chemical composition of fresh RJ changes with time and that moisture increases rapidly between 24 and 48 h and slower until 72 h after grafting. This is in agreement with previous studies (Mitsui et al., 1964; Lercker et al., 1984; Brouwers et al., 1987). The proportion of 10-HDA and protein followed an inverse trend when compared to moisture in fresh RJ, indicating a dilution effect (addition of water, addition of jelly with higher water content, or passive water vapour absorption through hygroscopic effect). This was confirmed by the estimation of the proportion of 10-HDA and proteins in the dehydrated samples that remained constant. In contrast, Liu et al. (2008) found the proportion of 10-HDA of 24 h RJ was significantly lower than those of 48 h RJ but higher than 72 h RJ (Liu et al., 2008), indicating possible geographical or genetic differences between honeybee populations for this variable. The decrease in protein content shown in this study is comparable to other results (Brouwers et al., 1987; Messia et al., 2005). Sugar content of fresh RJ decreased on the first day and stabilised during day two and three. This again is linked to dilution since sugar content calculated in the dehydrated samples remained stable over the 72 h as was also found by Brouwers (1987). In previous studies, acidity was reported to increase during storage of RJ (see Chen and Chen, 1995), whereas we observed a significant decrease during the first three days.

According to Liu et al. (2008), SOD activity of 24 h RJ is in most cases significantly higher than 48 h RJ and 72 h RJ (Liu et al., 2008). However, our results showed SOD activity of fresh RJ harvested 24, 48 and 72 h after grafting did not change significantly, indicating that 24 h RJ does not possess higher antioxydative activity based on superoxide radicals. In fact, taking into account the decreasing protein content from the first to third day, the value of SOD activity per gram protein of 24 h RJ is significantly lower than 48 h RJ and 72 h RJ. The difference of the results between the two studies might be due to an insufficient sample size of Liu et al. (2008), a different sample

Table III. Classification matrices of the discriminant analyses of royal jelly on wet and dry basis. The numbers of samples classified into specific categories by the model are shown in the table, as well as the percentage of cases correctly classified.

Wet basis	Predicted classification	24 h RJ	48 h RJ	72 h RJ	% Correct classification
Observed classification	24 h RJ	25	4	0	86
	48 h RJ	0	21	8	72
	72 hR J	0	7	22	76
	Total	25	32	30	78
Dry basis					
Observed classification	24 h RJ	22	4	3	76
	48 h RJ	4	15	10	52
	72 h RJ	5	7	17	59
	Total	31	36	30	62

preparation or real differences between RJ production by different lineages of bees. SOD activity therefore does not seem to represent a reliable freshness marker over a three day period. In contrast, furosine generated by the Maillard reaction seems to be a better indicator in the first days after grafting (Messia et al., 2005).

Comparison of the values measured here with the currently available quality standards (China (General Administration of Quality Supervision, Inspection and Quarantine of the People's Republic of China, 2002) and Switzerland (Schweizerisches Lebensmittelbuch, 2003)) shows that Early Harvest RJ's mainly conform to the standards (Fig. 2). There are however a few divergences: in several samples the proportion of sugars for 24 h old RJ's was above those allowed by Chinese standards. Since the Swiss standard has a wider range of values for sugars, 24 h RJ conforms with it. In contrast, the lower value of the range of Swiss standards for water content is placed too high to incorporate the lower content of 24 h old RJ. When the composition values of Early Harvest RJ's of other studies are included in the comparison, it appears that the protein ranges of Chinese and Swiss standards are too narrow. We found values for 24 h old RJ above the standards' maxima. In addition, the minimal value of the Chinese standards could be slightly overestimated since Liu et al. (2008) found values below the range (<11%) for 3 days old RJ's (Liu et al., 2008). Although higher protein and lower water content values certainly increase the quality of RJ, the range of standards should be adjusted to take them into account. The same is true for sugars that are present in higher proportions in Early Harvest RJ's. Current ranges could be widened to include the values of Early Harvest RJ's with the drawback of decreasing the overall quality. Alternatively, different ranges could be established for RJ harvested at different times.

Our analyses suggest that RJ collected earlier than 72 h has a good quality as far as the compounds measured in our study are concerned. Whether the lower productivity due to the lower amount of RJ deposited in the cells 24 h after grafting and the extra amount of work needed to harvest more often is compensated by a higher quality of the product remains to be ascertained.

From a biological point of view, despite a rather constant composition of RJ during the first days of queen larval development (Brouwers et al., 1987), larvae reared in the laboratory and fed with 24 h RJ, 48 h RJ and 72 h RJ revealed developmental differences. More queen-like individuals developed on 48 h RJ than on 24 h RJ and 72 h RJ (Mitsui et al., 1964) suggesting the importance of the water content of the royal jelly or that of variations in chemical composition not identified here. For example, the trend of titration acidity was different to that of the proportion of 10-HDA in the three kinds of RJ indicating that differences could occur in other acids.

As the therapeutic and other beneficial properties of royal jelly are being

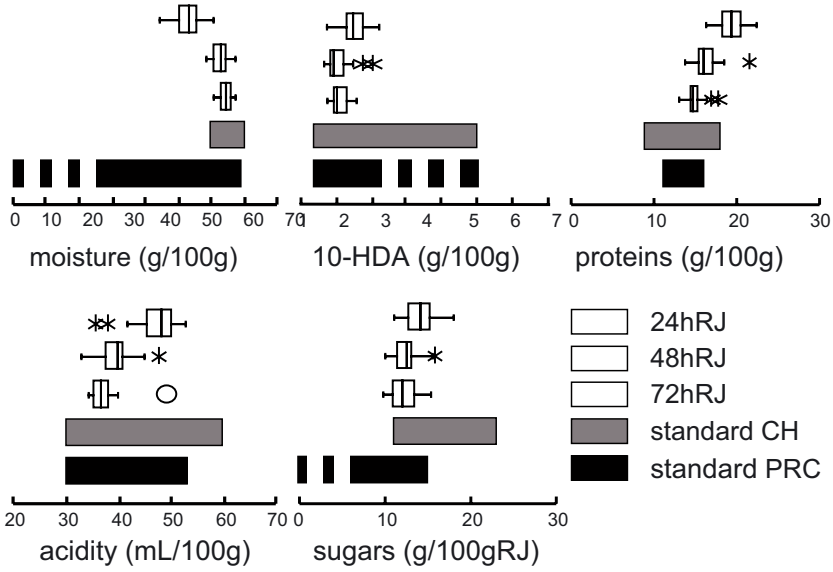

Figure 2. Comparison between the composition of RJ harvested at different times after grafting with the Chinese (PRC) and Swiss (CH) quality standards. RJ's harvested 24, 48 and 72 h after grafting are shown in order from top to bottom. The boxes represent first quartiles and third quartiles, the whiskers show the range of observed values that fall within the range 1.5 × first quartile–1.5 × third quartile, the stars and circles show the outliers and extreme outliers respectively. There are no superior limits to the Chinese range of 10-HDA and no inferior limits to the range of moisture and total sugar quantities.

characterised scientifically, the international market and trade of RJ is developing rapidly. Currently, the various product formulations developed are however put on the market without adequate standards for the control of their quality. Here we showed that time of harvest does affect the composition of RJ and some of the major constituents of RJ harvested earlier than 72 h were out of the range specified by quality standards. We suggest that existing quality standards of RJ should be revised to include the effect of harvest time on composition and that future international standards take this factor into account.

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Changements dans la composition de la gelée royale récoltée à différents intervalles de temps : conséquences sur les standards de qualité.

abeille / gelée royale / composition chimique / standards de qualité

Zusammenfassung – Veränderungen in Gelée royale in Abhängigkeit vom Erntezeitpunkt: Konsequenzen für Qualitätsstandards. Gelée royale (GR) ist ein Sekretionsprodukt der Hypopharynx- und Mandibeldrüsen von Ammenbienen und wird spezifisch an Königinnenlarven verfüttert. Es besitzt viele physiologische Aktivitäten und wird weltweit zu medizinischen und kosmetischen Zwecken und als Gesundheitsnahrung vermarktet. Die meisten Untersuchungen über die Zusammensetzung und Eigenschaften von GR, sowie die entsprechenden Qualitätsstandards basieren auf kommerziell verfügbarem GR, das im allgemeinen drei Tage (72 h) nach dem Umlarven gesammelt wurde. In China sammeln einige GR-Produzenten bereits einen (24 h) oder zwei Tage (48 h) nach dem Umlarven und werben mit Frische als Verkaufsargument. Es gibt jedoch kaum Daten zur Qualität von GR, das weniger als 72 h alt ist. Die vorliegende Untersuchung vergleicht

die chemische Zusammensetzung von GR, das 24, 48 und 72 h nach dem Umlarven gesammelt wurde, im Hinblick auf Feuchte-, Protein-, Gesamtzucker- und 10-HDA-Gehalt, sowie Säurewert und Superoxiddismutase(SOD)-Aktivität. Die Analyse von GR-Proben von 32 Völkern zeigte, dass die Zusammensetzung variiert für frische und dehydrierte Proben, und einige dieser lagen jenseits der für China und die Schweiz gültigen Standards. Demenstprechend sollten Angaben über den Erntezeitpunkt bei der Erstellung neuer Qualitätsstandards berücksichtigt werden.

Honigbiene / Gelée royale / chemische Zusammensetzung / Qualitätsstandard

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