JOURNAL OF
AGRICULTURAL AND
FOOD CHEMISTRY

Storage Stability Assessment of Freeze-Dried Royal Jelly by Furosine Determination

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The effect of freeze-drying and the assessment of the storage stability of freeze-dried royal jelly (RJ) were investigated by the determination of furosine and blocked lysine. The level of furosine in the RJ samples collected from cells at different times (1, 2, and 3 days after grafting) showed that the Maillard reaction had already occurred in the hive as indicated by the increase in furosine: from 9.6 to 20.8 mg/100 g of protein. Freeze-dried RJ was more prone to the early stage of the Maillard reaction than fresh RJ, as confirmed by the significantly higher furosine values found after 12 months, both at 4 °C (253.4 versus 54.9 mg/100 g of protein) and at room temperature (884.3 versus 332.5 mg/100 g of protein). After 18 months at room temperature, the lyophilized samples reached a furosine level of 1440.4 mg/100 g of protein, which corresponded to the blocked lysine levels, amounting to 24% of total lysine.

KEYWORDS: Royal jelly; freeze-drying; furosine; storage stability; protein

INTRODUCTION

Royal jelly (RJ) is a secretion from the hypopharingeal and mandibular glands of worker bees (*Apis mellifera* L.). RJ is a most interesting healthy and functional ingredient and food because it possesses several health-promoting and pharmacological properties (*1*, *2*). However, RJ may spoil and lose its health-promoting properties during processing and when improperly stored.

Raw, or natural, RJ is stored at 4 or −20 °C, whereas the lyophilized type, either alone or in combination with other ingredients, is generally sold in capsulate or tablet form and is stored at cool or room temperature.

The average recommended storage time, for natural RJ, is 18 months under refrigeration and may be extended to 24 months for products stored at −17 °C. After being defrosted and packaged, the product should not be kept in a refrigerator for more than 12 months (*1*). Donadieu (*3*) recommends an optimal conservation temperature for natural RJ of 0−5 °C, which will preserve its efficacy for one year.

Recently Marconi et al. (*4*) demonstrated the reliability of furosine as a marker of the freshness and storage conditions of raw RJ. The extensive availability of reducing sugars (glucose and fructose) (*5*, *6*) and α and ϵ amino groups of amino acids and proteins (*7*, *8*) enables the formation of Amadori compounds during RJ storage at room temperature (*4*). The furosine assay is the most sensitive and most accepted method for assessing the formation of Amadori compounds during the early Maillard reaction (*9*−*11*). Furosine was successfully used to assess the freshness, shelf life, and storage conditions of different foods such as liquid and powdered milk (*11*−*13*), in-shell, pasteurized, and powdered eggs (*14*, *15*), both powdered infant formulas and cereals (*16*, *17*), jams and fruit-based foods (*18*), and honey (*19*).

To extend its shelf life, RJ can be freeze-dried. Freeze-drying is the drying process that best maintains the natural characteristics of the product because it does not damage or denature the thermolabile components (*1*). After freeze-drying, the RJ becomes extremely hygroscopic and must be protected from humidity by being stored in an airtight container.

Some authors (*3*, *20*) are of the opinion that freeze-dried RJ can be stored at room temperature for several years without deteriorating. However, there are very few in-depth studies on the changes that occur in RJ when freeze-dried and during the subsequent storage period.

Therefore, the aim of this work is to assess the storage stability of freeze-dried RJ by the assessment of furosine marker to verify the effectiveness of freeze-drying in extending the shelf life of RJ stored at room temperature and to guarantee consumers that the native properties of the product are maintained.

MATERIALS AND METHODS

Samples. *Freshly Harvested RJ.* RJ samples were obtained from the "Piana miele" beekeeper (Bologna, Italy) in May and June 2002. A Doolittle method for rearing queens was followed (*21*) using two different types of grafting: in the first case, the larvae were settled on a layer of residual RJ diluted with water (a common method used in the production of RJ), whereas in the other, only water was used. At 1, 2, and 3 days (when the greatest amount is produced) (*5*) after

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10.1021/jf0404647 CCC: $30.25 © 2005 American Chemical Society
Published on Web 05/07/2005

grafting, the queen cells (20 on the first day and 10 on the second and third days) containing RJ were removed from the frame and their wax tops pinched shut. They were immediately wrapped in aluminum foil and taken to the laboratory. The wax at the opening of the cell was removed and the cell opened to expose the queen larva and the RJ. The larva was removed and the RJ extracted with a microspatula, weighed (expressed as milligrams per cell), and then pooled with all of the RJ obtained from the cells that day. The RJ samples were immediately analyzed for protein and furosine quantification.

Freeze-Dried RJ. A freshly harvested RJ sample (150 g) collected from a large number of queen cells, 3 days after grafting (commercial harvesting time), obtained from the "Carrelli" beekeeper (Campobasso, Italy), was used. The RJ sample was carefully homogenized at room temperature and immediately analyzed for moisture, water activity (a_w), protein, and furosine and used as the control sample (time 0). The RJ sample was divided into two subsamples of 70 g each. One subsample was freeze-dried using a laboratory scale freeze-dryer device (model Moduly 0) from Edwards High Vacuum International (Crawley, U.K.), operating at −50 °C for 24 h. The freeze-dried and fresh RJ samples were placed into airtight glass vials (0.5 and 8.0 g, respectively) and stored at both room temperature and 4 °C for 18 months. Sampling was carried out in duplicate at 0, 1, 2, 4, 6, 8, 10, 12, and 18 months.

Analysis. *Moisture.* The determination of moisture was carried out using official AOAC method 925.45D (*22*). One gram of fresh RJ was added to desiccated quartz sand and mixed thoroughly. The sample was dried at <60 °C under ≤50 mmHg (6.7 kPa) pressure. Its weight was checked at 2 h intervals toward the end of the drying period until the weight fell to ≤2 mg.

Water Activity. The water activity (a_w) in fresh and freeze-dried RJ samples was measured at room temperature as vapor pressure at the dew point, using an Aqualab CX-2 instrument (Decagon Devices, Pullman, WA).

Protein. Protein content (N × 6.25) was determined according to the Dumas combustion method, AOAC method 992.23 (*22*), using a Leco nitrogen determiner (model FP-528, Leco Corp., St. Joseph, MI). An amount of 0.20 g of RJ sample was weighed in tinfoil (Leco tinfoil cups 502-186-100), using a foil holder (Leco no. 604-493), and twisting the ends of the foil to form a teardrop-shaped packet.

Furosine. An aliquot of sample (∼350 mg for fresh RJ and ∼100 mg for freeze-dried RJ), corresponding to ∼30−70 mg of protein, was hydrolyzed under nitrogen with 8 mL of 8 N HCl at 110 °C for 23 h. After hydrolysis, 0.5 mL of the hydrolysate was purified on a Sep-Pak cartridge (Waters Corp., Milford, MA), diluted, and determined according to the HPLC procedure devised by Resmini et al. (*23*).

A Waters HPLC system (Milford, MA) consisting of two pumps (model 510), a diode array detector (model 991), and an injector with a 50 μL loop (model 9125-080; Rheodyne, Cotati, CA) was used. Analytical separation was performed with an Alltech furosine-dedicated column (Deerfield, IL) at 280 nm wavelength. The furosine standard was purchased from Neosystem Laboratoire (Strasbourg, France).

Lysine. A RJ sample corresponding to 25 mg of protein was placed in a flask with an acid-resistant rubber stopper. After the addition of 25 mL of 6 N HCl, the flask was introduced under vacuum to an oven at 110 °C for 24 h. The flask was then cooled, and the sample was evaporated to dryness under gentle vacuum and dissolved in 0.1 N HCl. Lysine was determined by high-performance anion-exchange chromatography with pulsed amperometric detection (HPAEC-PAD), using a Dionex system (Dionex Corp., Sunnyvale, CA) composed of a gradient pump (model GP50) with on-line degas and an electrochemical detector (model ED40). Separation was performed by an AminoPac PA10 analytical column, 250 × 2 mm, with 8.5 μm particle size (Dionex). The quantitative determination of lysine was carried out by isocratic elution, using 50 mM NaOH with a flow rate of 0.25 mL/min, as reported by Marconi et al. (*4*).

Percent Blocked Lysine. The percent blocked lysine was indirectly calculated from the furosine values, because the Amadori compound, after 8 N acid hydrolysis, generates a molar yield of 42.5% of lysine and 43.4% of furosine, as reported by Krause et al. (*24*). From the furosine and lysine values, determined according to the above-mentioned chromatographic methods, was calculated the percent of blocked lysine:

$$\%\ \text{blocked lysine} = \text{blocked lysine} \times 100/(\text{blocked lysine} + \text{available lysine}) \quad (1)$$

In eq 1

$$\text{blocked lysine} = (1/0.434 \times \text{furosine})(\text{MW of lysine}/\text{MW of furosine})$$

and

$$\text{available lysine} = \text{chromatographed lysine} - (0.425 \times \text{blocked lysine})$$

Statistical Analysis. Results were expressed as mean values ± standard deviations of three determinations for freshly harvested RJ sample and of four determinations (two determinations for two separate aliquots) for storage test samples.

RESULTS AND DISCUSSION

Freshly Harvested RJ. The protein, furosine, and quantity of RJ removed 1, 2, and 3 days after grafting are reported in **Table 1**. The amount of RJ produced increased from 30 to 100 mg/cell on the first day to ∼200 mg/cell on the second day and to ∼500 mg/cell on the third day. Lercker et al. (*5*) found RJ amounts of 16.8 (3.4−37.3), 185.0 (116.5−288.4), and 398.2 (366.5−431.2) mg/cell for RJ removed 1, 2, and 3 day after grafting, respectively. Howe et al. (*25*) found values of 237 (109−389) mg/cell for RJ harvested on the third day after grafting.

The trend in protein content is very interesting during the 3 days of RJ production. It decreased constantly from the first to the third day both in cells grafted with RJ (from 14.7 to 11.8%) and in those grafted with water (from 16.2 to 11.0%). Hydroxy acid showed a similar trend (*26*), whereas the opposite was found in the glycidic fraction, as it increased as the larvae grew (*6*). These compositional changes could be explained by the biophysiologic needs connected with larva growth (*8*). With regard to the extent of the early Maillard reaction during RJ production, the high furosine value (31.1 mg/100 g of protein) found in the RJ removed 1 day after being grafted with residual RJ could be due to the low quality of the RJ used for grafting and the bees producing only a small amount of new RJ (∼100 mg/cell). In fact, the drastic reduction between the first and second days of production in samples grafted with RJ (from 31.1 to 13.9 mg/100 g of protein) was due to its dilution with significant amounts of freshly produced RJ (200 mg/cell). The increase in furosine on the third day of production may be attributed to the Maillard reaction, brought about by the high temperatures reached in the beehive during the RJ production season (June and July). The furosine value of the sample removed 1 day after grafting confirms this. It was grafted only with water and was remarkably lower (9.6 mg/100 g of protein) but had increased to 20.8 mg/100 g of protein by the third day.

The furosine level of 20.8 mg/100 g of protein, which was found in the RJ samples from cells harvested 3 days after grafting (commercial harvesting), could be taken as a reference level for assessing the freshness, shelf life, and state of conservation of raw RJ.

Freeze-Dried RJ. A comparison of some of the physicochemical properties of freeze-dried and fresh RJ is given in **Table 2**.

The freeze-drying process produced a significant reduction in product weight of ∼66%, confirming the findings of Karaali et al. (*20*) (70%). In fact, the level of moisture in the freeze-dried RJ fell from 67.2 to 3.4% and water activity from 0.97 to 0.40. The moisture content of freeze-dried RJ agrees with literature data ranging from 2.66 to 5.70% (*20*, *27*).

Freeze-drying slightly increased the Maillard reaction as the furosine value confirmed, varying from 18.0 mg/100 g of protein

Table 1. Furosine and Protein Levels of Raw Royal Jelly Collected 1, 2, and 3 Days after Grafting from Queen Cells Grafted with Royal Jelly or Water

days after grafting	amount of RJ (mg/cell)			furosine (mg/100 g of protein)		protein (% wb, N × 6.25)	
	RJ graft		water graft	RJ graft	water graft	RJ graft	water graft
	min–max	mean	mean	mean ± SD	mean ± SD	mean ± SD	mean ± SD
1	56.9–146.0	101.1	30.0	31.1 ± 0.45	9.6 ± 0.19	14.7 ± 0.09	16.2 ± 0.08
2	125.7–276.9	217.1	180.0	13.9 ± 0.67	13.6 ± 1.93	12.6 ± 0.18	13.3 ± 0.05
3	381.5–595.5	496.2	500.3	18.0 ± 1.13	20.8 ± 0.37	11.8 ± 0.08	11.0 ± 0.07

Table 2. Physicochemical Properties of Freeze-Dried and Raw Royal Jelly

	freeze-dried RJ	raw RJ
moisture (%)	3.4 ± 0.04	67.2 ± 0.10
water activity (a_w)	0.40 ± 0.00	0.97 ± 0.00
furosine (mg/100 g of protein)	24.6 ± 0.61	18.0 ± 1.04
lysine (g/100 g of protein)	7.67 ± 0.08	7.89 ± 0.15

Table 3. Furosine Level (Milligrams per 100 g of Protein) of Freeze-Dried and Raw Royal Jelly Stored at 4 °C and Room Temperature (RT) for 18 Months

storage duration (months)	freeze-dried RJ		raw RJ	
	4 °C	RT	4 °C	RT
0	24.6 ± 0.61		18.0 ± 1.14	
1		220.5 ± 16.28	18.0 ± 0.70	45.0 ± 0.41
2		338.1 ± 3.72	22.1 ± 0.21	117.6 ± 15.58
4		428.2 ± 7.58	25.0 ± 1.03	179.0 ± 8.12
6	95.1 ± 5.11	545.9 ± 9.63	36.2 ± 3.65	224.2 ± 17.11
8		605.4 ± 11.48	41.1 ± 2.37	264.3 ± 9.08
10		663.7 ± 1.69	45.3 ± 2.01	280.8 ± 8.33
12	253.4 ± 4.01	884.3 ± 18.18	54.9 ± 2.03	332.5 ± 6.17
18		1440.4 ± 10.74	63.5 ± 1.02	487.4 ± 18.06

of fresh RJ to 24.6 mg/100 g of protein of the freeze-dried sample due to the prolonged process (24 h) and to the manipulation of the product. However, the increase of furosine is very low because the freeze-drying is based on the principle that, under high vacuum, frozen water can be removed from a foodstuff without having to go through a liquid phase and high temperatures.

The effects of storage conditions (duration and temperature) on the development of the Maillard reaction in freeze-dried RJ and in the reference sample of freshly harvested RJ (3 days after grafting) are shown in **Table 3**.

The lyophilized RJ was more prone to Maillard reaction than fresh RJ. In fact, the furosine values for freeze-dried RJ stored at room temperature had already risen after 2 months (338 mg/100 g of protein) and, after 18 months, had reached considerably high values (1440.4 mg/100 g of protein). They were significantly higher than those obtained for raw RJ (117.6 and 487.4 mg/100 g of protein, respectively) that was stored under the same conditions.

The damage to the nutritional properties of RJ, assessed by calculating blocked lysine (also called biologically unavailable lysine) (*24*), highlighted that the damage to raw RJ stored for 18 months at 4 °C was limited (blocked lysine = 0.9% of total lysine), whereas it was very significant in the lyophilized samples stored for 18 months at room temperature (blocked lysine = 24% of total lysine) (**Figure 1**).

These findings are in contrast with the current opinion that freeze-dried RJ can be stored at room temperature for several years without deterioration (*1*, *3*, *20*). Karaali et al. (*20*) stated that RJ was more stable when lyophilized; however, their deduction was exclusively based on the assessment of titratable acidity (mainly due to lipid and protein hydrolysis). They found that the rates of change in acidity of lyophilized products stored at room temperature were almost the same as that of natural RJ stored at refrigerated temperatures.

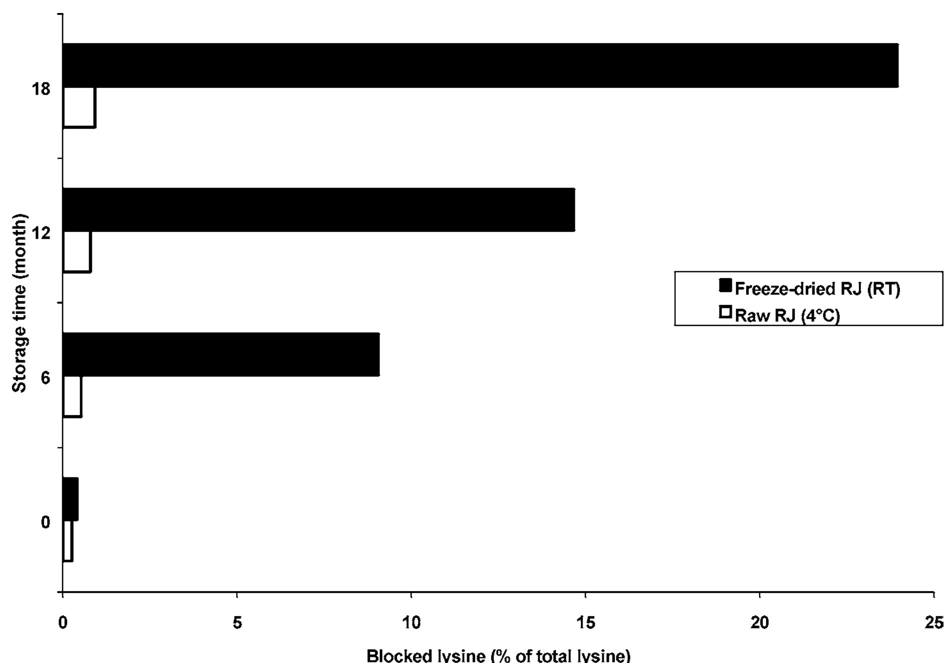

Figure 1. Blocked lysine of freeze-dried and raw RJ stored for different times at room temperature (RT) and 4 °C, respectively.

The greater susceptibility of freeze-dried RJ to the Maillard reaction is also confirmed with refrigerated storage (4 °C): the level of furosine was higher in the freeze-dried RJ than in the raw sample after both 6 and 12 months (95.1 versus 36.2 mg/100 g of protein and 253.4 versus 54.9 mg/100 g of protein, respectively).

These results could be explained by the fact that during freeze-drying the increasing concentration of solids produces interactions between sugars and proteins and decreases water activity (fresh RJ $a_w = 0.97$, freeze-dried RJ $a_w = 0.40$), making the product more susceptible to the Maillard reaction (*16*, *28*, *29*).

In addition, the above-mentioned data confirm that a storage temperature of 4 °C is appropriate for raw RJ (furosine and blocked lysine were 54.9 g/100 g of protein and 0.8% after 12 months and 63.5 g/100 g of protein and 0.9% after 18 months, respectively), as already demonstrated by Marconi et al. (*4*). Their findings showed that when a commercial RJ sample was stored for 10 months at 4 °C at the beginning of its shelf life, furosine content increased only slightly from 72.0 to 100.5 mg/100 g of protein.

In conclusion, the nutritional properties of RJ undergo minor damage during the freeze-drying process, but freeze-dried RJ is highly susceptible to MR and therefore does not meet the product quality and stability requirements. Therefore, the statement that freeze-drying is a valid process for extending the shelf life of RJ needs to be revised. Nevertheless, furosine is an appropriate parameter to assess the quality of natural/raw and lyophilized RJ as well as the effectiveness of various processes and storage conditions.

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Received for review November 16, 2004. Revised manuscript received March 18, 2005. Accepted March 20, 2005. An abridged version of this paper was presented at the V Congresso Nazionale di Chimica degli Alimenti "Qualità e Sicurezza degli Alimenti" (Fifth National Congress on Food Chemistry, "Food Quality and Safety"), June 9−12, 2003, Parma, Italy.

JF0404647