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Bioactive compounds and health-promoting properties of royal jelly: A review

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ABSTRACT

Royal jelly (RJ) is an important functional food item that possess several health promoting properties. It has been widely used in commercial medical products, healthy foods and cosmetics in many countries. RJ has been demonstrated to possess numerous functional properties such as antibacterial activity, anti-inflammatory activity, vasodilative and hypotensive activities, disinfectant action, antioxidant activity, antihypercholesterolemic activity and antitumor activity. Biological activities of RJ are mainly attributed to the bioactive fatty acids, proteins and phenolic compounds. In consideration of potential utilisation, detailed knowledge on the composition of RJ is of major importance. The diversity of applications to which RJ can be put gives this novel food great industrial importance. This review summarises the composition, nutritional value and functional properties of RJ.

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Abbreviations: RJ, royal jelly; MRJPs, RJ proteins; ELISA, enzyme-linked immunosorbent assay; HPLC, high-performance liquid chromatography; FAA, free amino acid; TAA, total amino acid; AMP, adenosine monophosphate; ATP, adenosine triphosphate; ADP, adenosine diphosphate; 10-HDA, 10-hydroxy-2-decenoic acid; HDAA, 10-hydroxydecanoic acid; FA, fatty acids; GC-MS, gas chromatography–mass spectrometry

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1. Introduction

Recent years have seen growing interest on the part of consumers and the food industry into functional food ingredients and the ways in which it may help maintain human health. The important role that diet plays in preventing and treating illness is widely accepted. The basic concepts of nutrition are undergoing a significant change. The classical concept of “adequate nutrition,” that is, a diet that provides nutrients in sufficient quantities to satisfy particular organic needs, is being replaced by the concept of “optimal nutrition,” which includes, besides nutrients, the potential of food to promote health, improve general well-being and reduce the risk of developing certain illnesses. This is where functional foods, also known as designer foods, therapeutic foods, superfoods, or medicinal foods, play their part (Nagai & Inoue, 2004). The market for functional foods is increasing at an annual rate of 15–20% (Hilliam, 2000). A functional food may be natural or be obtained by eliminating or modifying one or more of its basic components. Some components may also be added to food to make them “functional” among them ω 3 fatty acids, vitamins, probiotics, fibre, phytochemicals, bioactive peptides and phytosterols may be named (Chandrasekara & Shahidi, 2011; De La Rosa, Alvarez-Parrilla, & Shahidi, 2011). Among foods that possess potential health benefits are those originating from the beehive, namely honey, propolis and royal jelly.

Royal jelly (RJ) is one of the most attractive functional foods that have for sometime been a commercial product, especially in dietetics and cosmetics, in many countries. RJ is a thick and milky secretion from the hypopharyngeal and mandibular glands of young worker bees (*Apis mellifera* L.) and is used to feed the larvae (Isidorova, Czyzewska, Isidorovab, & Bakier, 2009). The queen honeybee is fed RJ throughout the larval period, while nurse honeybees are fed RJ for only 3 days (Simuth, 2001; Srisuparb, Klinbunga, Wongsiri, & Sittipraneed, 2003).

Many studies on biological activities as well as chemical composition of RJ have been conducted. Given the exceptional biological properties attributed to it, RJ has considerable commercial appeal and is today utilised in many sectors, ranging from the pharmaceutical and food industries to the cosmetic and manufacturing sectors. This has resulted in large-scale importation in countries where production is insufficient to meet domestic demand. Research capabilities thus need to be reinforced to permit both a reliable qualitative and quantitative evaluation of the different components and the implementation of analytical tests on commercially available products.

Commercially, RJ can be produced in significant quantities using the Doolittle queen-rearing technique. No official data exist about the RJ market as of yet (Grillenzoni, 2002), but China is unanimously acknowledged as being the leading world producer and exporter of RJ, which it sells at a highly competitive price. Chinese production of RJ is estimated at 2000 tonnes/year (a quantity that represents over 60% of production worldwide), almost all of which is exported to Japan, the United States and Europe. Other countries like Korea, Taiwan and Japan are important producers and also exporters. Elsewhere in the world, RJ is produced mainly in Eastern Europe and to a lesser extent in Western Europe and particularly

in Mexico (Sabatini, Marcazzan, Caboni, Bogdanov, & de Almeida-Muradian, 2009).

Numerous studies have been dedicated to RJ since the late 19th century (Lercker, 2003; Planta, 1888). However, it is difficult to bring together the data collected by different authors due to the lack of homogeneity among the materials used, the different sampling procedures and production conditions. Additional complicating factors are the multiplicity of experimental conditions, as well as the diversity of the analytical methods used and their continued evolution. Knowledge of the composition of recently produced RJ is essential in order to define a standard composition, evaluate the quality of commercial products and detect the presence of RJ in other products which containing it. At present some countries, like Switzerland (Bogdanov et al., 2004), Bulgaria, Brazil (Brasil, 2001) and Uruguay have defined national standards for this product.

2. Composition and quality criteria for royal jelly

RJ is a viscous jelly substance which is partially soluble in water with a density of 1.1 g/mL. Its colour is whitish to yellow, the yellow colour increases upon storage. Its odour is pungent, the taste being sour and sweet. The sensory characteristics are important quality criteria for RJ. Old RJ, which has not been properly stored, tends to be darker and possess a rancid taste. For optimum quality, RJ should be stored in frozen state. The viscosity of RJ varies according to water content and age and it slowly becomes more viscous when stored at room temperature or in a refrigerator at 5 °C. The increased viscosity appears to be related to an increase in water insoluble nitrogenous compounds, together with a reduction in soluble nitrogen and free amino acids. These changes are apparently due to continued enzymatic activities and interaction between the lipid and protein fractions. Physical characteristics RJ appear as a whitish substance with a gelatinous consistency, often not homogenous due to the presence of undissolved granules of varying sizes. It has a distinctively sharp odour and taste. It is partially soluble in water and highly acidic (pH 3.4–4.5) with a density of 1.1 g/mL (Lercker, 2003). There are no RJ international standards. However, some countries like Brazil, Bulgaria, Japan and Switzerland have established national standards.

Table 1 shows the composition of fresh and lyophilised RJ (Sabatini et al., 2009). The data originate from investigators in many countries and under different regulations, but for the establishment of general standards, further investigations are needed. Due to the product's high water content, compositional characteristics are also proposed for the freeze-dried sample. This enables a more direct comparison of data as RJ is marketed in this form. Although the overall analytic data confirm that exposure to a temperature of 4 °C causes no alterations in RJ composition, it has also been shown that only storage of RJ in frozen state prevents decomposition of biologically active RJ proteins and thus RJ should be frozen as soon as it is harvested (Li, Wang, Zhang, & Pan, 2007).

The composition of RJ is quite complex. It contains different proteins, amino acids, organic acids, steroids, esters,

Table 1 – Composition of fresh and freeze-dried RJ.

| | Fresh RJ | Freez-dried RJ |
|-----------------------------|----------|----------------|
| Water (g/100 g) | 60–70 | <5 |
| Lipids (g/100 g) | 3–8 | 8–19 |
| 10-HDA (g/100 g) | >1.4 | >3.5 |
| Protein (g/100 g) | 9–18 | 27–41 |
| Fructose (g/100 g) | 3–13 | – |
| Glucose (g/100 g) | 4–8 | – |
| Sucrose (g/100 g) | 0.5–2.0 | – |
| Ash (g/100 g) | 0.8–3.0 | 2–5 |
| pH | 3.4–4.5 | 3.4–4.5 |
| Acidity (mL 0.1 N NaOH/g) | 3.0–6.0 | – |
| Furosine (mg/100 g protein) | <50 | – |

phenols, sugars, minerals, trace elements and other constituents. In addition, the composition of RJ varies with seasonal and regional conditions. In the 1980s a workgroup was held in Italy which devoted much attention to the composition of RJ (Antinelli et al., 2003; Boselli, Caboni, Sabatini, Marcazzan, & Lercker, 2003; Lercker, Caboni, Conti, Ruini, & Giordani, 1981, 1982; Lercker, Caboni, Vecchi, Sabtini, & Nanetti, 1993; Lercker, Vecchi, Piana, Nanetti, & Sabtini, 1984a; Lercker, Vecchi, Sabtini, & Nanetti, 1984b; Lercker et al., 1985, 1986; Vecchi, Sabatini, Grazia, Tini, & Zambonelli, 1988). The composition of the main constituents of RJ, proteins, carbohydrates and lipids is widely reported in the literature (Bonomi et al., 1986; Garcia-Amoedo & Almeida-Muradian, 2007; Lercker, 2003; Pourtallier, Davico, & Rognone, 1990; Takenaka & Echigo, 1980). Chemically, fresh RJ comprises water (50–70%), proteins (9–18%), carbohydrates (7–18%), fat (3–8%), mineral salts (ca. 1.5%) and small amounts of polyphenols and vitamins. The lyophilised product contains <5% water, 27–41% protein, 22–31% carbohydrate and 15–30% fat.

The values obtained by the various authors are fairly in agreement, notwithstanding the high variability displayed for some parameters (sugars and lipids). It should be kept in mind that the reported findings refer to different number of samples taken in different places and at different times of production and that different methods of sampling and analysis were used. Moreover, RJ is naturally non-homogeneous. Analyses of RJ samples of different geographical origins showed no difference in composition in order to distinguish one product from another. It may similarly be affirmed that environmental conditions do not significantly influence the main components (Sabatini et al., 2009).

2.1. Water

Water content of RJ is fairly uniform and greater than 60%, and a water activity above 0.92, nonetheless RJ displays considerable microbial stability. The constancy of the moisture content is basically assured, inside the hive, by the continuous provision of fresh supplies of this substance by nurse bees, by the natural hygroscopicity of RJ and the entire colony's efforts to maintain a level of ambient moisture; moreover the insolubility of some compounds can explain the variations in water content (Sabatini et al., 2009).

2.2. Carbohydrates

Carbohydrates accounts for about 30% of the dry matter of RJ. However, while the components are highly constant in qualitative terms, considerable variability exists from a quantitative standpoint. As in honey, the monosaccharides fructose and glucose are the main sugars. Depending upon the age and beekeeping foods, glucose constituted 50–70% of the total sugars (Chen & Chen, 1995). Together, fructose and glucose often account for over 90% of the total sugars. Sucrose is always present but often in highly variable concentrations (Lercker et al., 1986). It is also possible to find oligosaccharides such as trehalose, maltose, gentiobiose, isomaltose, raffinose, erlose and melezitose; though present in very small concentrations, they are useful for identifying a characteristic pattern, which is comparable to that of honey and in some cases indicative of the authenticity of the product (Sabatini et al., 2009).

2.3. Proteins, peptides and amino acids

Proteins (27–41%) represent the most important portion of the dry matter of RJ. More than 80% of RJ proteins are soluble proteins (MRJPs) (Simuth, 2001). Eight major RJ proteins (MRJP1, MRJP2, MRJP3, MRJP4, MRJP5, MRJP6, MRJP7 and MRJP8) have been characterised by the cloning and sequencing of their respective cDNAs (Albert & Kludiny, 2004). MRJPs are thought to be the major factors responsible for the specific physiological role of RJ in queen honeybee development, as MRJPs include numerous essential amino acids, similar to ovalbumin and casein (Schmitzova et al., 1998). The MRJP family, which accounts for most of the soluble proteins (31%), had nine members; MRJPs 1–9 (Albert & Kludiny, 2004; Drapeau, Albert, Kucharski, Prusko, & Maleszka, 2006; Schonleben, Sickmann, Mueller, & Reinders, 2007). MRJP 1 is a weak acidic glycoprotein (pI 4.9–6.3, 55 kDa) and forms an oligomer that is estimated to be 350 or 420 kDa (Bilikova et al., 2002; Kimura et al., 2003; Simuth, 2001). MRJP2, MRJP3, MRJP4 and MRJP5 are estimated to be glycoproteins of 49, 60–70, 60 and 80 kDa, respectively (Schmitzova et al., 1998). MRJP 2–5 are mainly in the basic pI range (pI 8.3) (Li et al., 2007; Sano et al., 2004; Santos et al., 2005; Scarselli et al., 2005; Schonleben et al., 2007; Tamura, Kono, Harada, Yamaguchi, & Moriyama, 2009).

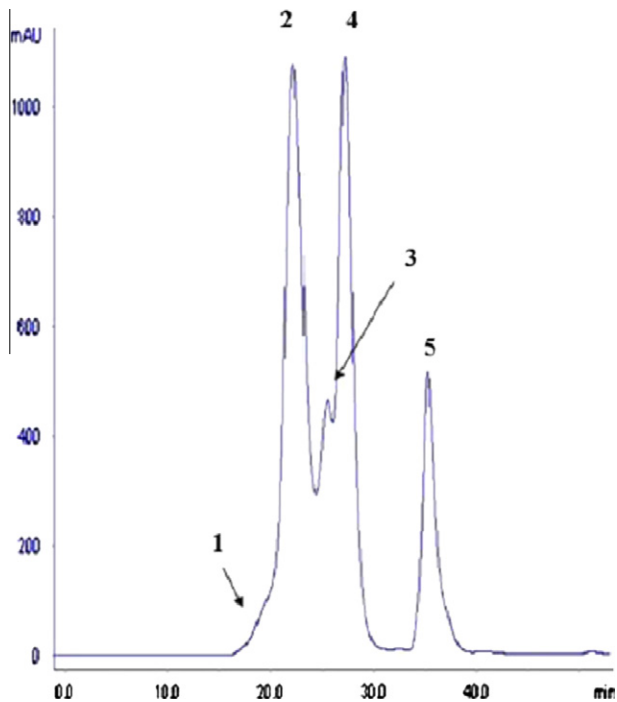


Fig. 1 – Chromatogram of elution profiles of crude soluble RJ proteins by size-exclusion HPLC on a Superose 12 column. The protein absorbance was monitored at 280 nm. Peaks: 1 (640 kDa), 2 (280 kDa), 3 (100 kDa), 4 (72 kDa) and 5 (4.5 kDa). Absorbance of the 280 kDa peak varied widely among RJ samples. (A) Elution pattern of 280 kDa protein rich RJ sample. Absorbance of 280 kDa peak and 72 kDa peak was 1075.632 mAU and 1088.532 mAU, respectively.

Soluble RJ proteins have been extracted by dialysis followed by several centrifugation techniques. Soluble RJ proteins are universally separated into five peaks (640, 280, 100, 72 and 4.5 kDa) by size-exclusion HPLC on a Superose 12 column (Fig. 1). Among these peaks, both the 280 and 72 kDa peaks were major, but the intensity of the 280 kDa peak differed markedly among original RJ samples. The main 280 kDa protein was separated into a 55 kDa band by reducing and non-reducing SDS-PAGE (Fig. 2). This protein was also separated into multiple spots ranging from pH 4.2–6.5 by 2-DE. MRJP1 was thought to comprise an oligomer complex linked by non-covalent bonds under natural conditions. Another major protein, the 72 kDa peak on Superose 12 HPLC, was identified as MRJP2 (Tamura et al., 2009).

Recently, Bilikova and Simuth (2010) developed enzyme-linked immunosorbent assay (ELISA) using specific polyclonal anti-apalbumin 1 antibody for determination of 55 kDa major protein of RJ, named apalbumin 1, which is an authentic protein of honey and pollen pellet. Gel-based and gel-free proteomic approaches and high-performance liquid chromatography (HPLC) chip quadruple time-of-flight tandem mass spectrometry were applied to comprehensively investigate the protein components of RJ (Han et al., 2011). Overall, 37 and 22 non-redundant proteins were identified by one-dimensional gel electrophoresis and gel-free analysis, respectively, and 19 new proteins were found by these two

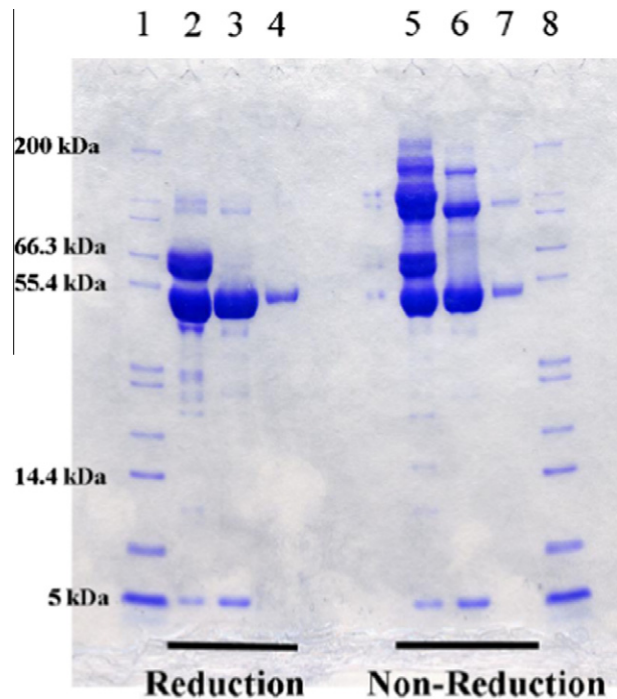


Fig. 2 – Reducing and non-reducing SDS-PAGE profiles of 280 kDa proteins. 1 and 8, molecular weight marker proteins using Mark 12 unstained standard kit (Invitrogen); 2, crude soluble RJ proteins with reduction; 3, 280 kDa proteins separated by Superose 12 HPLC with reduction; 4, main peak proteins separated by Mini Q HPLC with reduction; 5, crude soluble RJ proteins with non-reduction; 6, 280 kDa proteins separated by Superose 12 HPLC with non-reduction; 7, main peak proteins separated by Mini Q HPLC with non-reduction. The reduction procedure was performed with 50 mM DTT. The main 280 kDa protein was detected by a 55 kDa band with reducing and non-reducing conditions.

proteomic approaches. MRJPs were identified as the principal protein components of RJ, and proteins related to carbohydrate metabolism such as glucose oxidase, α -glucosidase precursor and glucose dehydrogenase were also successfully identified. Importantly, the 19 newly identified proteins were mainly classified into three functional categories: oxidation–reduction (ergic53 CG6822-PA isoform A isoform 1, Sec61 CG9539-PA and ADP/ATP translocase), protein binding (regucalcin and translationally controlled tumour protein CG4800-PA isoform 1) and lipid transport (apolipoprotein III-like protein).

Several studies have analysed MRJPs by reducing SDS-PAGE, including 2-DE (Li et al., 2007; Sano et al., 2004; Santos et al., 2005; Scarselli et al., 2005; Schmitzova et al., 1998; Schonleben et al., 2007). Separated bands on SDS-PAGE do not reflect the data for intact proteins, as samples are boiled and reduced as part of the standard Laemmli (1970) method. It was also reported that the 57 kDa protein deteriorates based on storage temperature or time (Kamakura, Fukuda, Fukushima, & Yonekura, 2001a; Kamakura, Suenobu, & Fukushima, 2001b), and this degradation of the 57 kDa protein is suppressed by ethylenediaminetetraacetic acid (EDTA) (Kamakura & Fukushima, 2002). It was therefore thought that

soluble RJ proteins deteriorate readily under natural conditions.

The physiological functions of the RJ protein have been reported. Crude RJ protein or separated soluble protein, including MRJPs, stimulates cell proliferation (Kamakura, 2002; Kamakura et al., 2001a, 2001b; Mishima et al., 2005; Narita et al., 2006; Watanabe et al., 1996, 1998). However, it has also been reported that crude RJ protein inhibits the bisphenol A-induced proliferation of human breast cancer cell lines (Nakaya et al., 2007). In addition, MRJP1 and MRJP2 stimulate mouse macrophages to release TNF- α (Simuth, Bilikova, Kovacova, Kuzmova, & Schroder, 2004), while MRJP3 modulates immune responses by suppressing the production of IL-4, IL-2 and IFN-c in T cells (Kohno et al., 2004). Okamoto et al. (2003) screened for antiallergic factors in RJ based on inhibition of IL-4 production by anti-CD3 stimulated spleen cells derived from OVA/alum-immunised mice. Using a series of column chromatographies, a 70 kDa glycoprotein, major RJ protein 3 (MRJP3) that suppresses IL-4 production was purified. In *in vitro* experiments, MRJP3 suppressed the production of not only IL-4 but also that of IL-2 and IFN-g by T cells concomitant with inhibition of proliferation. The MRJP3-mediated suppression of IL-4 production was also evident when lymph node cells from OVA/alum-immunised mice were stimulated with OVA plus antigen presenting cells. The purified suppressive factor on OVA/alum-induced allergic responses in mice was also examined. Interestingly, in spite of the antigenicity of MRJP3 itself as an extraneous foreign protein, intraperitoneal administration of MRJP3 inhibited serum anti-OVA IgE and IgG1 levels in immunised mice. In addition, heat processed soluble MRJP3 had reduced antigenicity while maintaining its inhibitory effects on antibody responses to OVA. The results indicated that MRJP3 can exhibit potent immunoregulatory effects *in vitro* and *in vivo*. Furthermore, considering the intriguing immunomodulatory effects of MRJP3, it may be of clinical significance to design MRJP3-derived antiallergic peptides by identifying the associated polypeptide regions.

It was recently found that protein and peptides has antioxidative activity (Kosińska, Karamać, Penkacik, Urbalewicz, & Amarowicz, 2011; Nagai & Inoue, 2004; Samaranayaka & Li-Chan, 2011; Shahidi & Zhong, 2011). In a recent study, 29 antioxidative peptides were isolated from RJ hydrolysate by membrane ultrafiltration, anion-exchange chromatography, gel filtration chromatography and reverse-phase high-performance liquid chromatography. The investigation focused on 12 small peptides with 2–4 amino acid residues: these structures were identified as Ala-Leu, Phe-Lys, Phe-Arg, Ile-Arg, Lys-Phe, Lys-Leu, Lys-Tyr, Arg-Tyr, Tyr-Asp, Tyr-Tyr, Leu-Asp-Arg, Lys-Asn-Tyr-Pro. Analysis of the antioxidative properties of these peptides revealed strong hydroxyl radical scavenging activity. Three dipeptides (Lys-Tyr, Arg-Tyr and Tyr-Tyr) containing Tyr residues at the C-terminal had strong hydroxyl-radical and hydrogen-peroxide scavenging activity. The antioxidant properties of these peptides were suggested to be due to a combination of their abilities to act as free-radical scavengers. Three tyrosyl dipeptides containing Tyr residues at their C-termini (Lys-Tyr, Arg-Tyr and Tyr-Tyr) have phenolic hydroxyl groups, which scavenge the free radicals via donation of a

hydrogen atom from their hydroxyl group (Guo, Kozuma, & Yonekura, 2009).

The principal nutritional value of protein food is determined by their amino acid content, and amino acid analysis has been successfully used for the analysis of proteins and peptides. Therefore, determination of the content changes of amino acids may be an effective way for accessing the quality of RJ. The amino acids present in the highest percentages were proline, lysine, glutamic acid, β -alanine, phenylalanine, aspartate and serine (Boselli et al., 2003). The concentration of series D amino acids was below the detection limit of the method (0.1 mg/g of RJ). No significant changes were observed in the overall concentration of free amino acids in RJ stored at 4 °C for 10 months. However, in the same samples stored at room temperature, the proline and lysine content showed an increase in the first three months and after 6–10 months decreased to levels slightly lower than those in the control. This suggests that, under favourable temperature conditions, proteolytic enzymatic activity continues to occur over time. Liming, Jinhui, Xiaofeng, Yi, and Jing (2009) developed a rapid ultra-performance liquid chromatographic (UPLC) method for feasible separation and quantification of 26 amino acids in RJ. The method was also applied to quantitatively determine free amino acid (FAA) and total amino acid (TAA) profiles in RJ samples stored at different temperatures for different periods. Results showed that the average contents of FAA and TAA in fresh RJ were 9.21 and 111.27 mg/g, respectively; the major FAAs were Pro, Gln, Lys, Glu, and the most abundant TAAs were Asp, Glu, Lys and Leu. Although the concentration of most FAAs and TAAs showed no significant difference during storage, contents of total Met and free Gln decreased significantly and continuously, and might be a parameter to predict the quality of RJ.

2.4. Adenosine

As a result of increasing interest in RJ with respect to human health, the authentication of active ingredients in RJ is becoming the subject of an increasing number of studies. Adenosine monophosphate (AMP) and adenosine monophosphate N1-oxide were found and identified in RJ (Noriko et al., 2006; Piana, 1996). Adenosine is a naturally occurring purine nucleoside and is formed by the breakdown of adenosine triphosphate (ATP). ATP is the primary energy source in cells for transport systems and the action of many enzymes. Most of the ATP is hydrolysed to adenosine diphosphate (ADP), which can be further dephosphorylated to AMP. If large concentrations of ATP are hydrolysed, some of the AMP can be further dephosphorylated to adenosine by the cell membrane associated with enzyme 50-nucleotidase (Enzo, Maria, & Luciano, 2001). The pathway to adenosine is shown in Fig. 3. Adenosine acts as a building block for nucleic acids and energy storage molecules, a substrate for multiple enzymes and an extracellular modulator of cellular activity (Alam, Szymusiak, Gong, King, & McGinty, 1999). The endogenous release of adenosine exerts powerful effects in a wide range of organ systems (Olah & Stiles, 1992, 1995). For example, adenosine has a predominantly hyperpolarising effect on the membrane potential of excitable cells, producing inhibition in vascular smooth

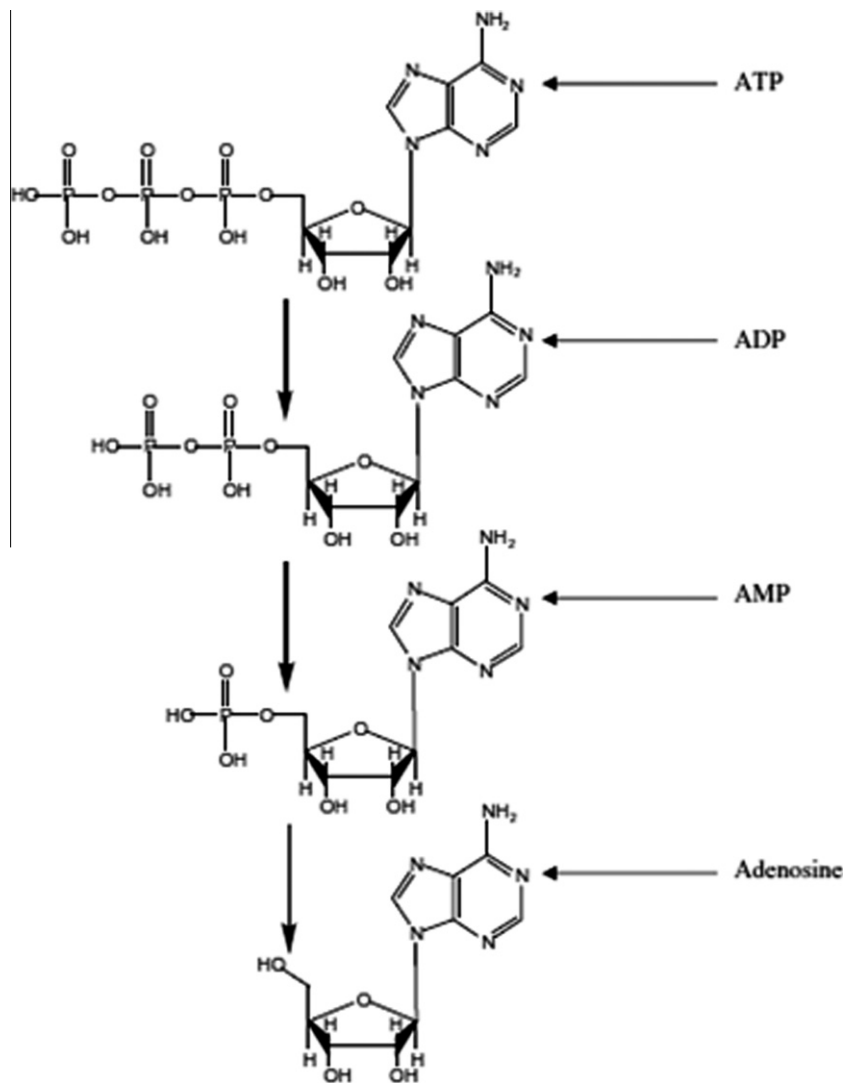


Fig. 3 – Pathway of adenosine.

muscle cells of coronary arteries and neurons in the brain (Basheer, Strecker, Thakkar, & McCarley, 2004). As an endogenous nucleoside, adenosine has been widely investigated in different products. For instance, adenosine is an important index for quality assessment of Lingzhi (*Ganoderma lucidum*) and Cordyceps (Gao et al., 2007; Gong, Li, Lia, Liu, & Wang, 2004). A simple method was described for the determination of adenosine in RJ. The adenosine in the sample was extracted using 80% ethanol and analysed by reversed-phase HPLC. For 45 RJ products, the adenosine content varied from 5.9 to 2057.4 mg/kg (Xue, Zhou, Wu, Fu, & Zhao, 2009). More recently, Kim and Lee (2011) determined the concentration of adenosine in pure RJ creams purchased in the United States

by HPLC. The results show that the concentration of adenosine lies between 27 and 50 $\mu\text{g/g}$ for pure RJ creams and between 2 and 173 $\mu\text{g/g}$ for RJ supplements.

2.5. Lipids, 10-hydroxy-2-decenoic acid (10-HDA) and organic acids

Fresh RJ comprises fatty acids (FA) and lipids (3–8%), while lyophilised product contains about 15–30% of fat. Chromatographic analysis showed that the chloroform/methanol extract obtained from RJ consists mainly of FA. The fast atom bombardment mass spectrum (FABMS) of this extract gave several ion peaks due to compounds with higher molecular weight than those of the FA so far reported. The methanol extract was found to contain unknown phospholipids (Noda, Umebayashi, Nakatani, Miyahara, & Ishiyama, 2005). The lipid composition is reported as 80–85% fatty acids, 4–10% phenols, 5–6% waxes, 3–4% steroids and 0.4–0.8% phospholipids. The FA fraction consists of 32% *trans*-10-hydroxy-2-decenoic acid (10-HDA, Fig. 4), 24% gluconic acid, 22% 10-hydroxydecanoic acid (HDA), 5% dicarboxylic acids and several other acids.

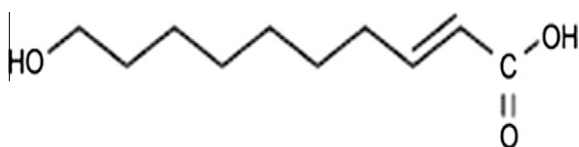


Fig. 4 – *trans*-10-hydroxy-2-decenoic acid (10-HDA).

10-HDA and HDAA are specific components of RJ (Terada, Narukawa, & Watanabe, 2011).

Unlike organic acids of most animal and plant materials, the fatty acids of RJ have 8–10 carbon atoms, and they are usually either hydroxy FA or dicarboxylic acids. Among them, a major FA was characterised as 10-HDA; it constitutes approximately 70% of the diethyl ether-soluble fraction (Noda et al., 2005). The unique feature of RJ is the set of 10-HDA, which is believed to be present only in RJ, is known for having various pharmacological effects (Isidorova et al., 2009). It was elucidated by Lercker et al. (1981) that the major FA of RJ consisted of 10-HDA and HDAA acids. For this reason, the 10-HDA content has been adopted as a marker for RJ and is used for RJ quality analysis. In the available literature few publications have investigated the FA composition of fresh and lyophilised RJ with the aid of gas chromatography–mass spectrometry (GC–MS) (Isidorova et al., 2009). Hydroxy acids with 10 carbon atoms (10-hydroxydecanoic and 10-hydroxy-2-decenoic acid) above all have also been identified as being responsible for important biological activities tied to the development strategies of the colony (Wu, Li, & Liu 1991). Six compounds were existed as novel mono- or di-esters of 10-hydroxy-2E-decenoic acid in which the hydroxyl group was esterified by another FA unit, and one was hydroxy-2E-decenoic acid 10-phosphate. In addition, 9-hydroxy-2E-decenoic acid existed as a mixture of optical isomers (Noda et al., 2005).

Antinelli et al. (2003) showed a 0.4% and 0.6% reduction in 10-hydroxy-2-decenoic acid in two RJ samples stored at room temperature for 12 months. It is difficult to evaluate such a reduction in a sample in the control phase. Moreover it is difficult to use 10-HDA decrease as a freshness marker because of its variable amount in fresh RJ. Both HPLC and electropho-

retic analysis of 10-HDA showed that samples of RJ from European origin contained smaller amounts of this compound; this evidence was confirmed by measuring total lipids after solvent extraction (Ferioli, Marazzan, & Caboni, 2007). Recently, Isidorova et al. (2009) identified thirty-five aliphatic acids by GC–MS from the ether extract of commercial preparations containing lyophilised RJ. More recently, Isidorov et al. (2011) stated that the unique feature of RJ is a set of C8, C10 and C12 hydroxy fatty acids. In all, 10 acids characteristic of this bee product were identified in different combinations in the analysed samples, namely: 7- and 8-hydroxyoctanoic, 3-hydroxydecanoic, 9-hydroxydecanoic, 9-hydroxy-2-decenoic, 10-hydroxydecanoic, 10-hydroxy-2-decenoic (10-HDA), 3,10-dihydroxydecanoic, 2-octene-1,8-dioic and 2-decene-1,10-dioic acids (Fig. 5). The higher relative abundance of these compounds was determined in genuine honeydew and healthier honeys, and in “herbal honeys” (23.8–40.8, 18.2–48.5 and 27.0–48.4 lg/g, respectively). Since RJ is known to have strong antibiotic efficacy, the results suggested that a part of the non-peroxide antibacterial activity of RJ might be of bee origin.

2.6. Minerals

Ash content represents 0.8–3% of RJ fresh matter (Garcia-Amoedo & Almeida-Muradian, 2007; Messia, Caboni, & Marconi, 2003). The major elements are, in descending order: K, Ca, Na, Mg, Zn, Fe, Cu and Mn (Benfenati, Sabatini, & Nanetti, 1986), present in specific ratios such as K/Na and Ca/Mg. The hypotheses regarding the quantitative presence of these metals have focused on factors outside the colony (environment, procurement of food and production period) and to some

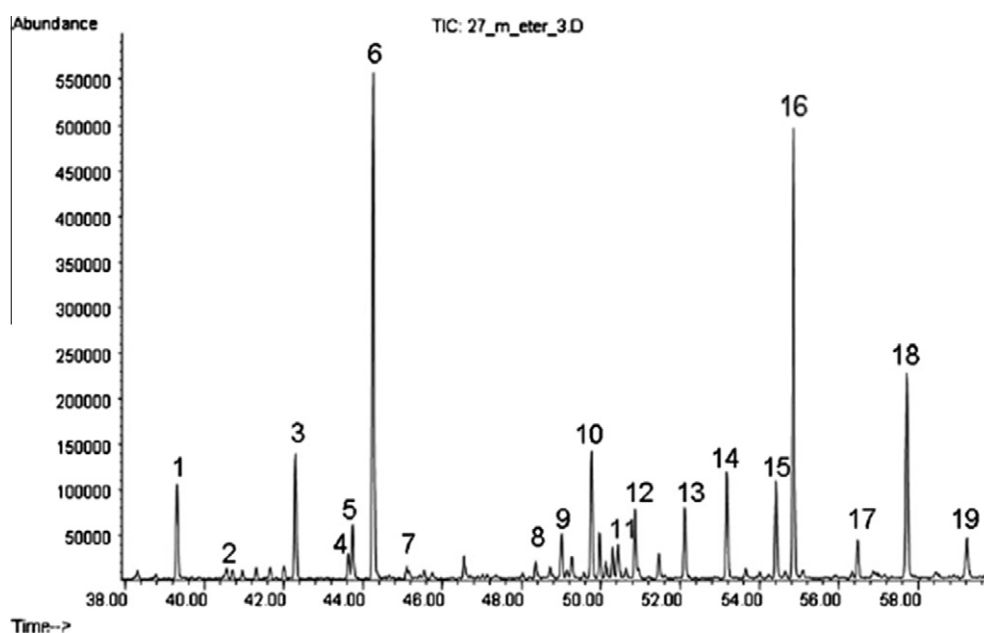


Fig. 5 – Chromatogram of carboxylic acids extracted from honeydew honey (HDH-5). (1) Salicylic acid, (2) 7-hydroxyoctanoic acid, (3) spheryllactic acid, (4) 8-hydroxyoctanoic acid, (5) 4-hydroxybenzoic acid, (6) 4-hydroxybenzeneacetic acid, (7) 3-hydroxydecanoic acid, (8) 2-octene-1,8-dioic acid, (9) 3,4-dihydroxyphenylethanol, (10) homovanillic acid, (11) 10-hydroxydecanoic acid, (12) protocatechuic acid, (13) 10-hydroxy-2-decenoic acid (10-HDA), (14) sebaccic acid, (15) p-coumaric acid, (16) 2-decene-1,10-dioic acid, (17) 3,10-dihydroxydecanoic acid, (18) hexadecanoic acid, (19) ferulic acid.

extent internal factors (biological factors tied to the bees). Trace elements play a key role in the biomedical activities associated with RJ, as these elements have a multitude of known and unknown biological functions. Concentrations of 28 trace (Al, Ba, Sr, Bi, Cd, Hg, Pb, Sn, Te, Tl, W, Sb, Cr, Ni, Ti, V, Co, Mo) and mineral (P, S, Ca, Mg, K, Na, Zn, Fe, Cu, Mn) elements were systematically investigated in botanically and geologically defined RJ samples (Stocker, Schramel, Kettrup, & Bengsch, 2005). Concentrations of K, Na, Mg, Ca, P, S, Cu, Fe, Zn, Al, Ba and Sr in RJ were determined by inductively coupled plasma optical emission spectroscopy (ICP-OES), while concentrations of Bi, Cd, Hg, Pb, Sn, Te, Tl, W, Sb, Cr, Mn, Ni, Ti, V, Co and Mo in RJ were determined by double focusing magnetic sector field inductively coupled plasma mass spectrometry (SF-ICP-MS). The concentrations of trace and mineral elements were quite constant in the associated RJ samples. The most important results were the homeostatic adjustments of trace and mineral element concentrations in RJ. This effect was evidently produced in the endocrine glands of nurse bees, which are adapted for needs of bee larvae. The study yielded a new finding that RJ, as a form of lactation on the insect level, shows the same homeostatic adjustment as mammalian and human breast milk.

3. Functional and health promoting properties

RJ is one of the most attractive ingredients for healthy foods. RJ is extensively used as cosmetic or dietary supplement due to the belief that it exerts similar effects on human beings as it does on honeybees. RJ has been demonstrated to possess several pharmacological activities in experimental animals, including vasodilative and hypotensive activities (Shinoda et al., 1978), increase in growth rate (Kawamura, 1961), disinfectant action (Yatsunami & Echigo, 1985), antitumour activity (Tamura, Fujii, & Kuboyama, 1987; Townsend, Brown, Felauer, & Hazlett, 1961), antihypercholesterolemic activity (Nakajin, Okiyama, Yamashita, Akiyama, & Shinoda, 1982) and anti-inflammatory activity (Fujii et al., 1990). In addition, antiaging (Iannuzzi, 1990; Inoue et al., 2003; Rembold, 1965), hypoglycaemic (Dixit & Patel, 1964; Fujii et al., 1990; Kramer, Tager, Childs, & Speirs, 1977) and antitumoral (Townsend et al., 1961; Townsend, Morgan, & Hazlett, 1959), are some of the properties attributed to RJ.

Chemical composition analysis has shown that RJ consists of a large number of bioactive substances such as 10-HDA, antibacterial protein (Fujiwara et al., 1990), a stimulating factor for the development of genital organs in male mice (Kato, Onodera, & Ishijima, 1988) and 350 kDa protein (Watanabe et al., 1998) that stimulates the proliferation of human monocytes. Therefore, RJ has been widely used in commercial medical products, health foods and cosmetics in many countries (Nagai, Sakai, Inoue, Inoue, & Suzuki, 2001).

Nagai and Inoue (2004) investigated functional and antioxidative properties of water extract (WSR) and an alkaline extract (ASR) prepared from fresh RJ. The yields were about 8.3% and 6.3% on a dry weight basis, respectively. Levels of total phenolic compounds were 21.2 and 22.8 $\mu\text{g}/\text{mg}$ RJ powder. Antioxidant activities, in both extracts, increased depending

on the concentration of the sample. The scavenging activities, against superoxide radical of WSR and ASR were high, and the activities at 100 mg/mL were the same as that of 5 mM ascorbic acid. This shows that the protein and phenolics fractions in RJ have high antioxidative activity and scavenging ability against active oxygen species. Many kinds of polyphenolic compounds, including flavonoids and cinnamic acid derivatives, are present in RJ, whose concentration depends on various factors, including plant species used by the bees, health of the plant, season and environmental factors (Gomez-Caravaca, Gomez-Romero, Arraez-Roman, Segura-Carretero, & Fernandez-Gutierrez, 2006; Kucuk et al., 2007). Principal flavonoids present in RJ include flavonoles (e.g., quercetin, kaempferol, galangin and fisetin), flavanones (e.g., pinocembrin, naringin and hesperidin) and flavones (e.g., apigenin, acacetin, chrysin and luteolin). It is well-known that the antioxidant properties of flavonoids could be predicted by investigating the availability of the phenolic hydrogens as the hydrogen donating radical scavengers according to their chemical structures (Buratti, Benedetti, & Cosio, 2007; Liu, Yang, Shi, & Peng, 2008).

Antitumour effects of RJ were investigated by employing transplantable tumours of mouse: advanced leukaemia L1210 and P388 strains and Ehrlich ascites, Sarcoma-180 ascites and solid tumours (Tamura, Fujii, & Kuboyama, 1985). The effects of RJ on tumour development and metastasis in murine tumour models was studied (Orsolich, Terzic, Sver, & Basic, 2005). Transplantable murine tumours were used: a spontaneous mammary carcinoma (MCA) and a methylcholanthrene-induced fibrosarcoma (FS) of CBA mouse. RJ did not affect the formation of metastases when given intraperitoneally or subcutaneously. However, synchronous application of tumour cells and RJ intravenously significantly inhibited the formation of metastases. These findings demonstrated that RJ products given orally or systemically may have an important role controlling tumour growth and metastasis.

Studies have shown different pharmacological activities of RJ, such as vasodilatory, metabolic, antimicrobial, anti-inflammatory, antioxidant and immunomodulatory effects (Fontana et al., 2004; Kohno et al., 2004; Liu et al., 2008; Nagai et al., 2001; Sver et al., 1996). *In vivo* experiments demonstrated both immunostimulatory and immunosuppressive effects of RJ, which could be the consequence of the presence of components possessing different immunomodulatory activities. Until now, most immunomodulatory effects of RJ have been ascribed to its protein components, especially to the major RJ protein 3 (Fang, Feng, & Li, 2010) and apalbumin 1 (Majtan, Kovacova, Bilikova, & Simuth, 2006). Major RJ protein 3 has been described as the dominant anti-inflammatory and immunosuppressive component of RJ which promotes anti-allergic response (Okamoto et al., 2003). In contrast, apalbumin 1, the major RJ and honey glycoprotein, exerts immunostimulatory and proinflammatory activities, by upregulating the production of tumour necrosis factor α (TNF- α) (Majtan et al., 2006).

Bioactivities of three RJ protein fractions (RJCP) were tested *in vitro* model systems (Salazar-Olivo & Paz-Gonzalez, 2005). RJCP stimulated cell growth of Tn-5B1-4 insect cells inducing 6.5 population doublings per mg of protein added to culture medium, meanwhile foetal bovine serum, the usual growth

supplement, gave rise only to 2.55 population doublings. RJCP and fractions RJP₃₀ and RJP₆₀ obtained by precipitation with ammonium sulphate, also affected Tn-5B1-4 cell shape and stimulated adhesion of these cells to the substrate. RJP₃₀ also increased the percentage of mature adipocytes in cultures of 3T3-F442A murine preadipocytes two-fold with respect to insulin treatment, without inducing additional cell growth of confluent preadipocytes. Fractions RJCP and RJP₆₀ showed similar capacity as that of insulin to stimulate the formation of mature 3T3 adipocytes. Fraction RJP₃₀ was also cytotoxic for HeLa human cervicouterine carcinoma cells, diminishing 2.5-fold the initial cell density after seven days of treatment. The presence of diverse bioactivities in RJ was shown to affect cell growth, cell differentiation and cell survival of insect, murine and human cancer cells. Gasic et al. (2007) and Vucevic et al. (2007) demonstrated that free short-chain fatty acids from RJ, containing 8–12 carbons, also exerted the modulatory activity on rat immune cells *in vitro*. The major fatty acid, 10-HDA, which is a unique component of RJ (Plettner, Slessor, & Winston, 1998), was predominantly immunosuppressive, as judged by the decreased proliferation of spleen T cells (Gasic et al., 2007) and the down regulation of co-stimulatory molecules and interleukin (IL)-12 production by spleen dendritic cells (DCs) (Dzopalic et al., 2011; Vucevic et al., 2007).

Jamnik, Goranovic, and Raspor (2007) investigated the anti-oxidative action of RJ in the cell of the *Saccharomyces cerevisiae* yeast as a model organism. At different time points cell energy metabolic activity was measured using the cell energy metabolism indicator resazurin, and dichlorofluorescein was applied to estimate intracellular oxidation. Results showed that RJ decreased intracellular oxidation in a dose dependent manner. Additionally, it affected growth and cell energy metabolic activity in a growth phase dependent manner. Protein profile analysis showed that RJ in the cell does not act only as a scavenger of reactive oxygen species, but it also affects protein expression.

RJ ameliorated the cisplatin (CP)-induced reductions in weights of testes, epididymides, seminal vesicles and prostate along with epididymal sperm concentration and motility. An increase in testes malondialdehyde (MDA) concentrations was detected, while significant decreases in superoxide dismutase, catalase and glutathione-peroxidase levels were noted in CP-alone group compared with the control group. The administration of RJ to CP-treated rats decreased the MDA level and increased superoxide dismutase, catalase and glutathione peroxidase activities in the samples (Silici, Ekmekcioglu, Eraslan, & Demirtas, 2009).

Some of pharmacologically active components in RJ possess immunomodulatory activity, but the mechanisms of their effect on the immune system have not yet been elucidated. The effect of 3,10-dihydroxydecanoic acid (3,10-DDA), a fatty acid isolated from RJ, on maturation and functions of human monocyte-derived dendritic cells (MoDCs) was studied (Dzopalic et al., 2011). It was shown that 3,10-DDA stimulated maturation of MoDCs by up-regulating the expression of CD40, CD54, CD86 and CD1a, and increased their allostimulatory potential in co-culture with allogeneic CD4⁺T cells. 3,10-DDA-treated MoDCs enhanced the production of IL-12 and IL-18, and stimulated the production of interferon- γ in co-culture with allogeneic CD4⁺ T cells, compared to MoDCs

control. In contrast, the production of IL-10 was down-regulated. The results suggested that 3,10-DDA stimulates maturation and Th1 polarising capability of human MoDCs *in vitro*, which could be beneficial for anti-tumour and anti-viral immune responses.

Terada et al. (2011) stated that activation of TRPA1 and TRPV1 induces thermogenesis and energy expenditure enhancement. They searched for novel agonists of TRPA1 and TRPV1 from RJ. The activation of human TRPA1 and TRPV1 by RJ extracts was measured and found that the hexane extract contained TRPA1 agonists. The main functional compounds in the hexane extract were *trans*-10-hydroxy-2-decenoic acid (HDEA) and 10-hydroxydecanoic acid (HDAA). Their EC₅₀ values were about 1000 times larger than that of AITC, and their maximal responses were equal. They activated TRPA1 more strongly than TRPV1. Their EC₅₀ values for TRPV1 were two times larger, and the maximal response was less than half of that for TRPA1. They also studied the potencies of other lipid components for both receptors; most of them had a higher affinity to TRPA1 than TRPV1. Among them, dicarboxylic acids showed equal efficacy for both receptors, but those were present in only small amounts in RJ. It was concluded that the main function of RJ was TRPA1 activation by HDEA and HDAA, the major components of the RJ lipid fraction.

4. Royal jelly contamination, adulteration and authenticity

Few studies concerning the possible contamination of RJ have been published. The content of RJ contaminants, compared to other bee products, was relatively low (Fleche, Clement, Zeggane, & Faucon, 1997). Melamine has become the focus of attention for the possible occurrence of nephrolithiasis and associated deaths, because it was added to foods to increase the apparent protein content by unethical manufacturers. Hydrophilic interaction chromatography/tandem mass spectrometry (HILIC-MS/MS) method was used for the determination of melamine in the RJ and RJ lyophilised powder (RJLP). The chromatographic separation was linear in the concentration range of 0.01–8 $\mu\text{g}/\text{mL}$ in RJ and 0.05–10 $\mu\text{g}/\text{mL}$ in RJLP for melamine (Zhou et al., 2009). Recently, the problem of RJ contamination by antibiotics has arisen. Although most studies concern residues in honey, antibiotic use in the colony can also contaminate RJ (Matsuka & Nakamura, 1990). On the other hand, it has been shown that RJ residue analysis is difficult and old analytical methods are questionable. There are few publications on antibiotic residues in RJ, mainly on chloramphenicol (Calvarese, Forti, Scortichini, & Diletti, 2006; Dharmananda, 2003; Reybroeck, 2003). The presence of chloramphenicol was detected in 29 out of 35 tested samples imported in Italy, the concentrations ranging from 0.6 to 28 $\mu\text{g}/\text{kg}$, with an average content of 6.1 $\mu\text{g}/\text{kg}$. As antibiotics are not allowed for use in beekeeping, there is no maximum residue level (MRL) for honey or other bee products in the European Union. For CA in honey the EU has established an MRL of 0.3 $\mu\text{g}/\text{kg}$. By using the method developed by Calvarese et al. (2006) this MPRL can also be used for RJ.

RJ adulteration is the most important quality problem. Adulteration by the nursing jellies for worker and drones is

improbable because of the very little amounts available for harvest. Adulteration with honey is more probable, causing an increase of the sugar values, the other values being lowered (Serra-Bonvehi, 1991). The most important quality criteria for RJ adulteration is 10-HDA. However, the composition limits reported in the literature are very broad. 10-HDA content decreases with storage of RJ (Antinelli et al., 2003). This decrease is higher in honey containing RJ (Matsui, 1988). Thus, the determination of all FAs, as carried out in Italian studies (Lercker et al., 1981, 1993), might be better approach for the determination of 10-HDA only. Adulteration with more than 25% yoghurt, egg white, water and corn starch slurry can be detected by the enhancement of moisture, diminishing lipid, protein and 10-HDA contents as well as the insolubility in alkaline medium (Garcia-Amoedo & Almeida-Muradian, 2007). Furthermore, microscopic analyses of RJ sediment, applied according to the basic principles of melissopalynology (Louveaux, Maurizio, & Vorwohl, 1978; Ricciardelli D'Albore, 1986) and in particular the identification of the pollens it contains, make it possible to define the geographical origins of the product and detect mixtures where they occur. Pollen identification is made easier by the fact that only a few countries actually produce RJ and specialists are capable of formulating their respective characteristic pollen associations. Another promising parameter for the evaluation of RJ authenticity is the presence of apalbumin (Simuth et al., 2004).

Authenticity of RJ production can be determined by measuring of the ratios of the stable isotopes of the C and N (Stocker, 2003). The authenticity of production can be measured by determining the FA composition of RJ (Howe, Dimick, & Benton, 1985; Lercker et al., 1993). The geographical authenticity can also be determined by pollen analysis (Ricciardelli d'Albore, 1986; Ricciardelli d'Albore et al., 1978). The amount of pollen, as well as visible wax and larvae particles should be minimal. RJ has a relatively low concentration of bacteria (Serra-Bonvehi & Escola-Jorda, 1991). The parameters investigated for the mentioned studies concern the organoleptic characteristics, physicochemical properties and the composition factors. The authenticity of honey and other honeybee products has long been evaluated on the basis of properties that can be defined by chemical, physical and instrumental analyses. Nowadays, it is generally understood that the quality of honeybee products should be defined in terms of physiological functions of their authentic components. Studies have revealed the importance of the lipid fraction as a marker and hence a criterion by which to determine the authenticity of the product. Presently, 10-HDA is mostly used for routine testing of RJ authenticity. The determination of the stable isotopes of C and N (Stocker, 2003) is also a promising approach for the determination of the authenticity of production. Adulteration of honey results in a general diminution of proteins and lipids and a relative increase of sugars (Serra-Bonvehi, 1991).

5. Freshness

RJ is a complex matrix that contains water (60–70%), crude protein (12–15%), carbohydrate (10–16%), lipid (3–7%), traces of mineral salts and vitamins. RJ may spoil or deteriorate

and lose its commercial value eventually when it is stored improperly, hence it should be stored at 4–8 °C or less to guarantee its good quality (Chen & Chen, 1995). Some quality and freshness evaluation indices, such as furosine (Messia, Caboni, & Marconi, 2005), superoxide dismutase (Chaozhong & Youlu, 1999), glucose oxidase and 57-kDa protein (Masaki, Toshiyuki, Makoto, & Masami, 2001) have been proposed. However, until now, few suitable physicochemical parameters as freshness and quality standards have been accepted worldwide.

Another fundamental aspect lies in the possibility of defining a parameter of RJ freshness. It has been noted that the macroscopic composition of RJ is fairly stable on the whole but also variable. Thus it is not a suitable parameter for defining product freshness. For the latter purpose, experiments were conducted on RJ samples stored at 4 and 20 °C over a period of 24 months to assess changes in the content of the enzyme glucose oxidase. The results obtained showed that the enzyme contained in RJ is influenced both by both storage temperature and time. At 20 °C it had decreased significantly after one month and degraded completely after one year. Even at 4 °C there was an evident, albeit modest, reduction in the enzyme. The determination of glucose oxidase is analytically very simple and thus within the capabilities of all laboratories (Boselli et al., 2002). Marconi, Caboni, Messia, and Panfili (2002) quoted several experiments were performed to evaluate the possibility of using furosine content as a marker for RJ freshness. Among the criteria for RJ quality analysis, 10-HDA content has been proposed as a freshness parameter (Antinelli et al., 2003).

The value of furosine, a product of Maillard's reaction, proved very low (from 0 to 10 mg/100 g of protein) in freshly produced RJ samples (Messia et al., 2003), but increased over time and in relation to temperature. Specifically, the content of rose to as high as 500 mg/100 g of protein after 18 months of storage at room temperature and 50 mg/100 g at 4 °C. Samples taken from store shelves showed values ranging from 40 to 100 mg/100 g protein. By contrast, freeze-dried RJ showed strong tendency to form furosine during storage (Messia et al., 2005).

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