Quality Assurance and Safety of Crops & Foods

ICC MoniQA

Quality Assurance and Safety of Crops & Foods 2011, **3**, 185–190

ORIGINAL ARTICLE

Metabolic fingerprinting of royal jelly: characterization and proof of authenticity

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Keywords
bee products, characterization, electrospray Ionization mass spectroscopy, proof of authenticity, royal jelly.

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Received 15 October 2010; Revised 29 March 2011; Accepted 6 July 2011

doi: 10.1111/j.1757-837X.2011.00112.x

Abstract

Introduction Metabolic fingerprint is a high-throughput screening to provide a sample classification. *Objectives* Herein, we describe a metabolic fingerpint strategy for proof of authenticity and possible adulteration of natural products. This strategy provides useful and complementary information to food science for content analysis. *Methods* Twelve samples of commercial royal jelly were analysed by electrospray ionization mass spectrometry (ESI-MS) in the negative mode. ESI-MS/MS was performed for characteristic negatively charged ions. *Results* Thermostability studies were also performed to royal jelly samples in order to evaluate the change on chemical composition with different times of exposure to heating and storage at room temperature. *Conclusion* The methodology developed in this work is useful to proof of authenticity and degradation of royal jelly samples using minimum sample preparation and direct injection of extracts.

Abdelnur PV, Abe S, Cunha IBS, Lima-Pallone JA, Godoy HT, Eberlin MN, Catharino RR (2011). Metabolic fingerprinting of royal jelly: characterization and proof of authenticity. *Quality Assurance and Safety of Crops & Foods*, **3**, 185–190.

Introduction

Royal jelly (RJ), a principal food of the honeybee queen, is produced by the hypo-pharyngeal and mandibular glands of worker honeybees. It has been reported that RJ has several pharmacological activities, including effect of the RJ on sperm quality in mice (Karacal & Aral, 2008), antitumor activity (Nakaya *et al.*, 2007; Izuta *et al.*, 2009), antihypercholesterolemic activity (Viuda-Martos *et al.*, 2008), anti-inflammatory activity (Kohno *et al.*, 2004), antihypertensive activity (Takaki-Doi *et al.*, 2009), antifatigue activity (Kamakura *et al.*, 2001) and antiallergy activity (Okamoto *et al.*, 2003). Analysis of chemical composition shows that RJ is composed mainly of proteins, sugars, lipids, vitamins and free amino acids, together with a large number of bioactive substances such as 10-hydroxy-2-decenoic acid (Olimpia *et al.*, 2008). Therefore, RJ has been widely promoted as a commercially available medicine, as a healthy food and as a cosmetic in many countries.

However, only a few studies have applied metabolic fingerprint strategy to characterize natural products (Mauri & Pietta, 2000; Roesler *et al.*, 2007; Gollucke *et al.*, 2009), and this strategy has proven to be versatile and can often be employed with only limited sample preparation to yield immediate compositional information of polar compounds. Metabolic fingerprint with direct insertion analysis has been used for complex samples mixtures such as soybeans (Santos *et al.*, 2006), ham (Moller *et al.*, 2007), cachaca (de Souza

et al., 2007), biodiesel (Catharino *et al*., 2007) and micotoxins (Catharino *et al*., 2005b).

Herein, we report metabolic fingerpint results using ESI-MS of methanolic extracts of four different species from the Labiatae family. The MS fingerprints recorded in positive and negative ion mode provide additional information to gas chromatography mass spectrometry studies (Isidorov *et al*., 2011), both for detailed studies of biomarkers and identification of particular components in essential oil or aroma extracts under investigation. In addition, it is shown that ESI-MS/MS of characteristic ions was performed to allow characterization of the species under investigation.

Materials and methods

Chemicals

Analytical high-performance liquid chromatography grade methanol and chlorophorm used for extraction as well as ammonium hydroxide were purchased from Merck SA (Rio de Janeiro, Brazil).

Materials

Three different brands of RJ samples commercially available were purchased from supermarkets in Brazil (São Paulo state) from January through April of 2007. Three lots were analysed, differentiated by fabrication dates for each of the four trademarks. All samples were within the validity period and without any visible damages. All determinations were conducted in duplicate.

Thermostability studies

The RJ samples were kept at a temperature of 60 °C and were collected after different periods of time. We analysed samples at zero time and after 1 and 48 h at room temperature, as well as after 1, 2, 3, 4 and 5 h of heating.

Extraction

The method used to extract compounds from the RJ was carried out according to the procedure previously described (Catharino *et al*., 2005a). The samples were extracted individually with chlorophorm, dried, and were dissolved in a solution containing methanol : water (1:1). In this method, the final amount of the sample is 0.25 mg mL^{-1} extraction solvent, and the extracts were kept in brown flasks at 5 °C until analysis, which was carried out within hours after preparation.

Mass spectrometry

A quadrupole time-of-flight mass spectrometer (Micromass, Manchester, UK) was used for fingerprinting ESI-MS analysis with an electrospray ion source. The instrument parameters were set to a source temperature of 100 °C, capillary voltage of 3.0 kV and cone voltage of 40 V for the positive ion mode. Measurements were also performed in the negative mode, ESI(-)-MS, and 10.0 μL of 0.5% NH_4OH was added to the sample mixture having a total volume of 1000 μl, yielding 0.1% as final concentration. In general, ESI-MS was performed by direct infusion with a flow rate of 10 μL min^{-1} using a syringe pump (Harvard Apparatus, Holliston, MA, USA). Mass spectra were acquired and accumulated over 60 s and mass range between *m/z* 50–1000. For collision induced dissociation, or tandem mass spectrometry (MS/MS), the ions of interest were selected by the quadrupole and directed to the collision cell. Argon was used as target gas and the energy of the ions were set in the range of 15–55 eV.

Data handling

All data obtained from ESI-MS of the various specie extracts were treated using MassLynx 3.5 (Waters, Manchester, UK). Mass spectra data was accumulated over approximately 20 s and the relevant mass range was selected and enlarged, which depending on MS mode, varied between *m/z* 50–550 or 900, respectively (a range that contained all ions of interest as judged by visual inspection).

Chemometrics analysis

Principal component analysis (PCA) was performed using the 2.60 version of Pirouette software from Infometrix (Woodinville, WA, USA). All the signals of intensity above 20% were considered for PCA.

Results and discussion

We have analysed four types of commercial pure RJ (AS, LAM, PL, PRO) as well as adulterated RJ (AS) with 100 μL of powered milk by ESI(-)-MS. In addition, thermostability studies were performed collecting RJ (AS) samples in different times after storage at room temperature and also after heating.

A quite similar mass spectra profile was obtained by four different classes of RJ (Figure 1), which means that their chemical composition was almost identical and that there is no significant variation in their composition. RJ composition

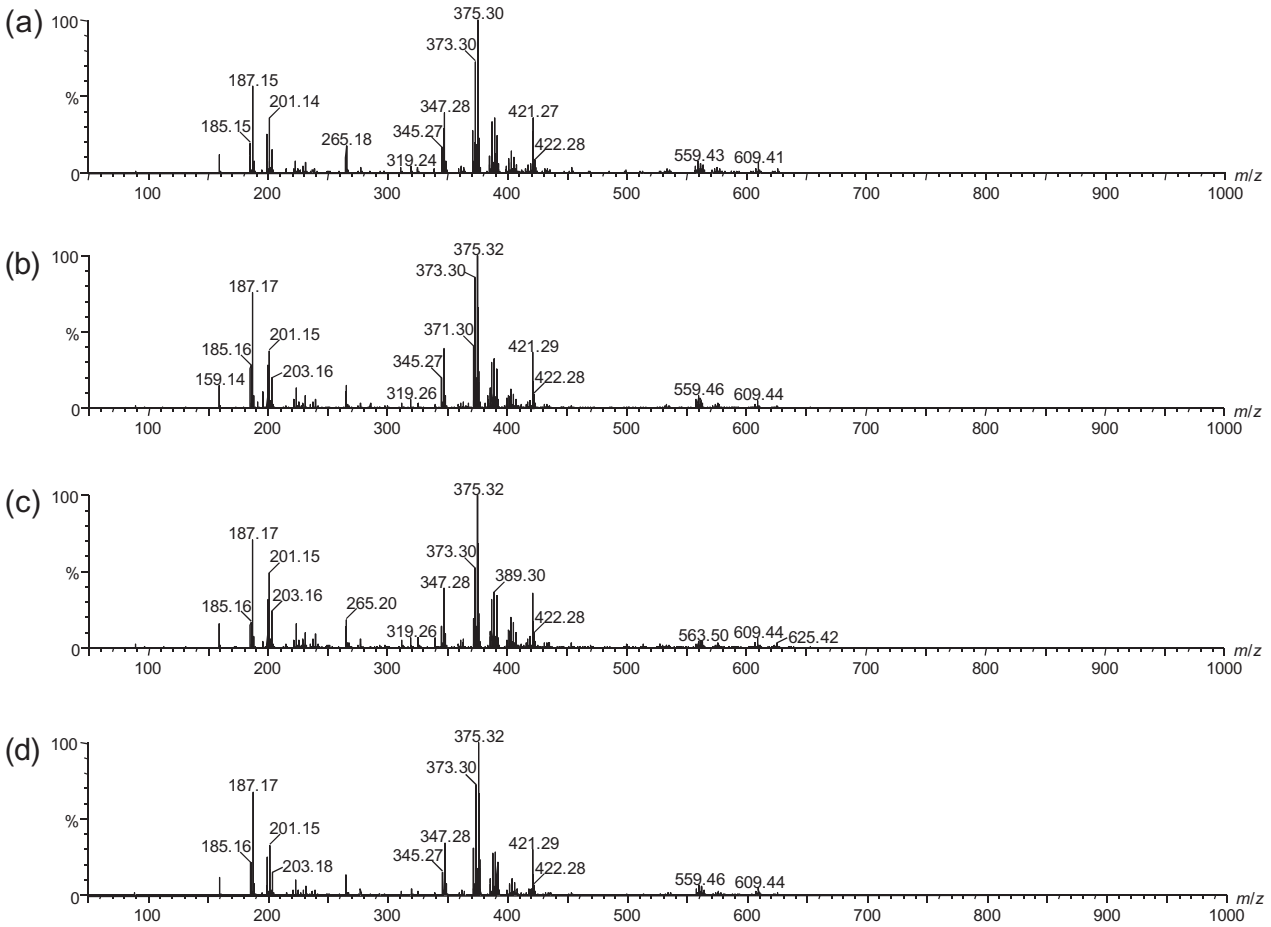

Figure 1 Electrospray ionization mass spectrometry spectrum of some fresh royal jelly brands: (a) AS, (b) LAM, (c) PL and (d) PRO.

has been studied (Olimpia *et al.*, 2008) and it is known to include lipids, sugars, fatty acids (FA) and others. The most common FA detected in RJ is 10-hydroxy-2*E*-decenoic acid (10-HDA) and it is considered an important constituent because of its pharmacological activities. Also detected was 8-hydroxy-octanoic, an unsaturated FA with the same number of carbons as 10-HDA (Noda *et al.*, 2005), both present in all samples analysed, the ions *m/z* 185 and *m/z* 187, respectively. High-resolution mass spectrometry and MS/MS experiments were performed to confirm the identity of these compounds (Figure 2). ESI(-)-MS/MS spectra showed the same fragmentation profile for both, which includes the neutral loss of formic acid or ethanol (46 Da), common dissociation pattern for FA.

The MS/MS of the ions *m/z* 199 and *m/z* 201 suggest that they refer to aldehyde compounds, since both had one loss of 44 Da (CH_3COH), characteristic loss for this class of compounds. The ion of *m/z* 201 suffers a water loss of 18 Da, which means that its structure has an additional hydroxyl group. Therefore, we believe that these ions correspond with $C_{12}H_{24}O_2$ and $C_{11}H_{22}O_3$ compounds, respectively. The last ion identified was *m/z* 265, which was previously described in an analysis of a RJ sample as 10-HDA phosphate compound (Noda *et al.*, 2005).

A completely distinct profile was detected analysing RJ samples adulterated with powdered milk (Figure 3). The main signals detected refer to sugar compounds, and these are present in high concentrationa in powdered milk, for example, the ion *m/z* 377 refers to chloride dissacharides, $[\text{cellobiose} + \text{Cl}]^-$ (Jiang & Cole, 2005), and could be used as a marker to this kind of adulteration. In addition to that, signals of FA are suppressed by the signals of the sugar compounds. This kind of adulteration is very common because the taste remains similar to pure RJ, and the whole process is cheaper and easier than to collect samples from beehive, and it does not depend on the season.

Thermostability studies were performed to verify if there is modification in the chemical composition of RJ samples

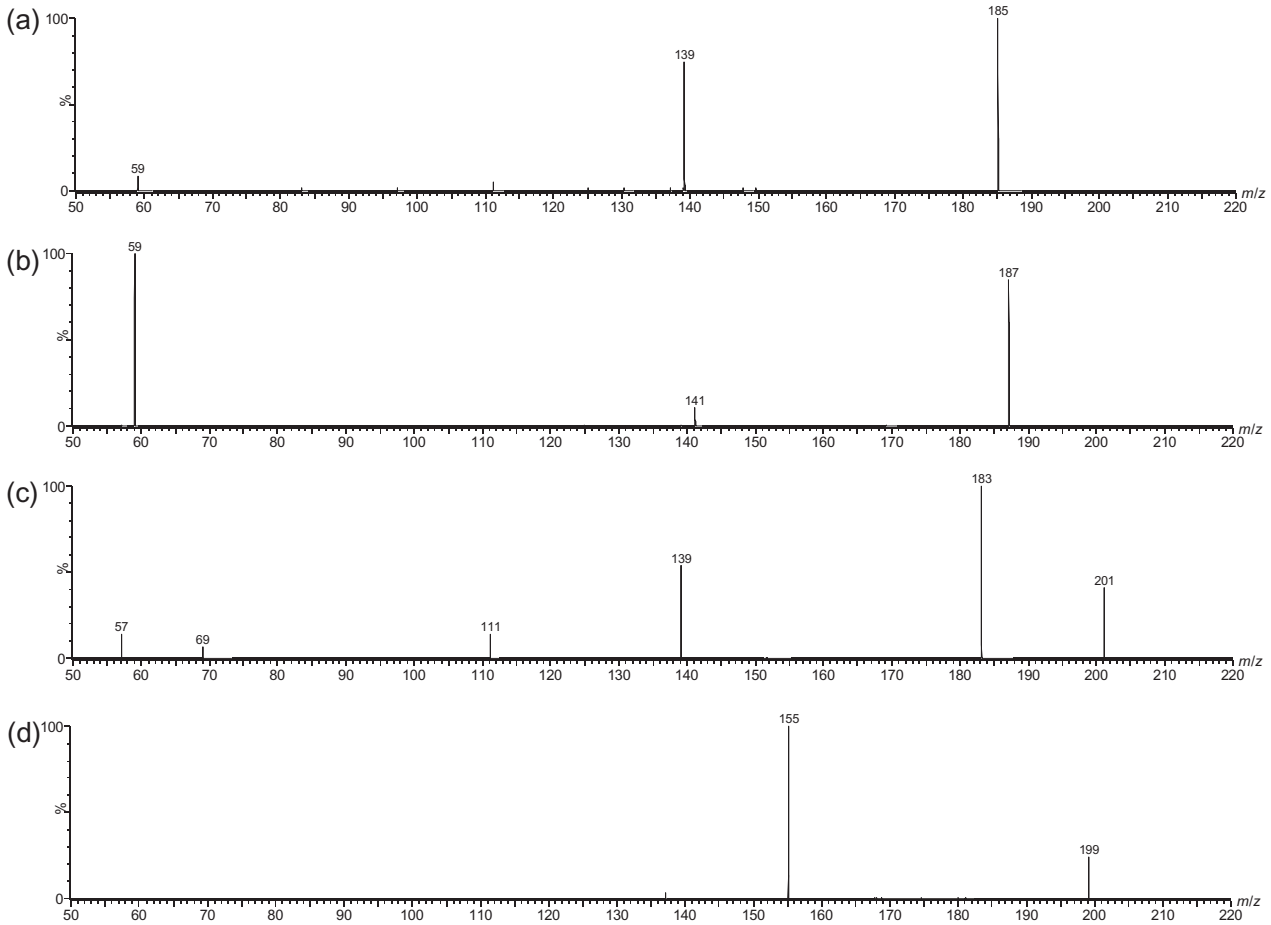

Figure 2 Electrospray ionization(-)-tandem mass spectrometry spectrum of ions *m/z* 185, 187, 199 and 201, respectively.

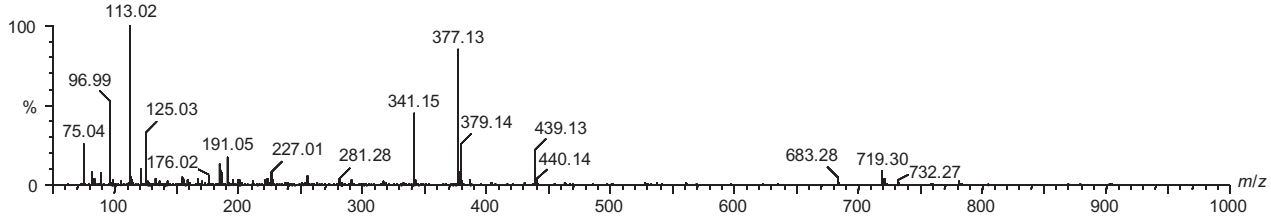

Figure 3 Electrospray ionization mass spectrometry spectrum of royal jelly (AS) adulterated with powered milk.

after removal from the beehive. This study was done to verify if some degradation process is occurring in royal jelly samples after being stored in market shelves for a long period of time.

The results show a sharp degradation caused by exposure to temperature as the full scan ESI(-)-MS spectrum shows. The same fingerprint was observed for the samples without temperature treatment (fresh samples), another fingerprint for 1–4 h with heating, another for 5 h with heating and another for 48 h without heating storage. Each temperature of sample exposure condition (0, 1–4, 5 and 48 h) resulted in a different chemical fingerprinting-MS. It is clearly noted that the RJ sample has a drastic modification in its composition after storage for 48 h, once a few signals were detected (Figure 4). It was not possible to detect 10-HDA phosphate after 48 h and signal intensities of the FA ions (*m/z* 185 and *m/z* 187) changed its ratio. With these results, we can infer that there is a change in the chemical profile of RJ after a period of time in which they are subjected to heat and storage, which means that

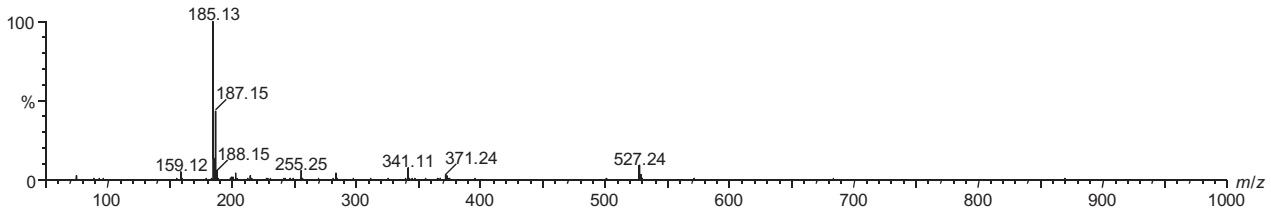

Figure 4 Electrospray ionization mass spectrometry spectrum of RJ 48-h storage at room temperature.

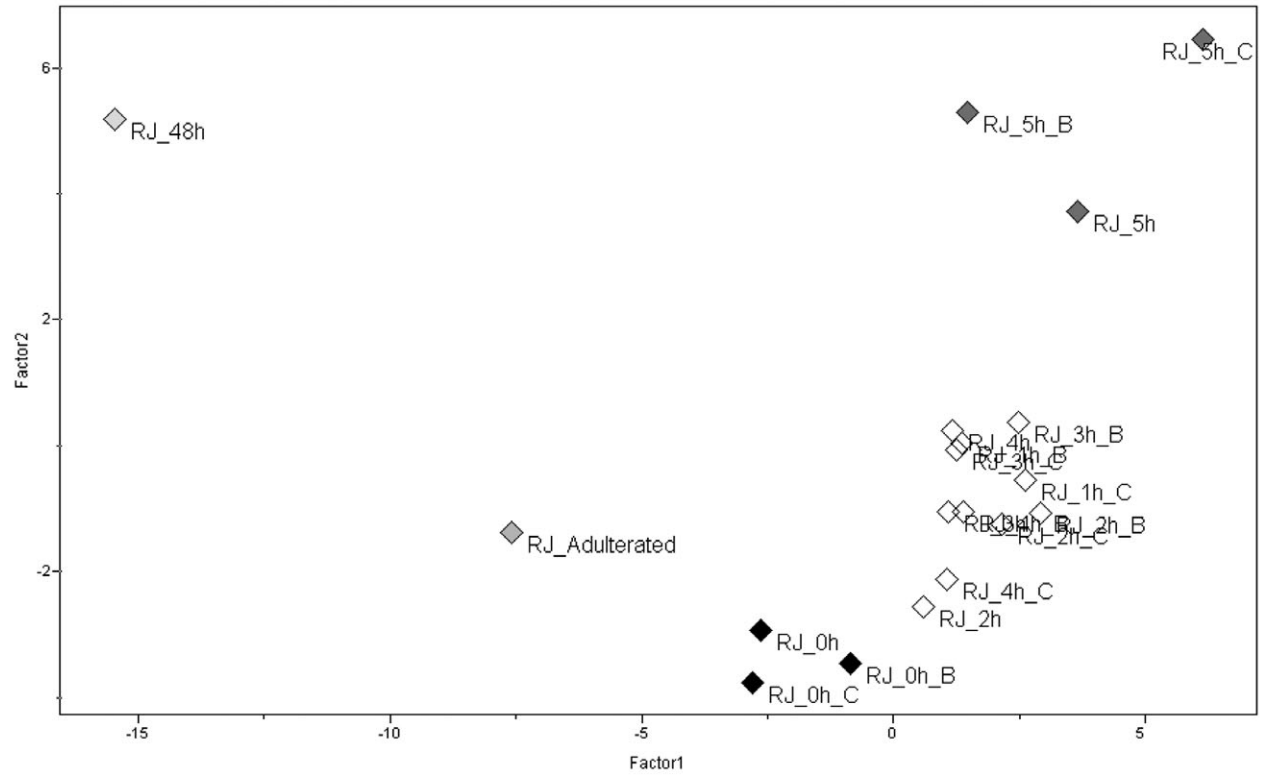

Figure 5 Principal component analysis of fresh, 1–5 h of heating and 48-h storage royal jelly (RJ) (AS) samples.

degradation occurred through the time and temperature of exposition.

All data from the fingerprint spectra were analysed by PCA, using Pirouette, confirming the visual evaluation of the results (Figure 5). The samples were divided in five groups with different profiles, which change with the increase of time that the samples were subjected to temperature and also with their purity.

We have studied the thermostability of RJ and concluded that there is degradation after time passed by and we could determine by some markers if the RJ is fresh, pure or adulterate using an easy and fast method by fingerprint direct infusion mass spectrometry. In only one analysis, we have the proof of authenticity as well as the main compound class present in the RJ, which proves it is fresh or degraded.

Acknowledgement

The authors would like to thank the Brazilian research Foundations FAPESP, CNPQ and CAPES for financial support.

References

Catharino R.R., Haddad R., Cabrini L.G., Cunha I.B.S., Sawaya A., Eberlin M.N. (2005a) Characterization of vegetable oils by electrospray ionization mass spectrometry fingerprinting:

classification, quality, adulteration, and aging. *Analytical Chemistry*, **77** (22), 7429–7433.

Catharino R.R., Marques L.D., Santos L.S., Baptista A.S., Gloria E.M., Calori-Domingues M.A., Facco E.M.P., Eberlin M.N. (2005b) Aflatoxin screening by MALDI-TOF mass spectrometry. *Analytical Chemistry*, **77** (24), 8155–8157.

Catharino R.R., Milagre H.M.S., Saraiva S.A., Garcia C.M., Schuchardt U., Eberlin M.N. (2007) Biodiesel typification and quality control by direct infusion electrospray ionization mass spectrometry fingerprinting. *Energy & Fuels*, **21** (6), 3698–3701.

de Souza P., Siebald H.G.L., Augusti D.V., Neto W.B., Amorim V.M., Catharino R.R., Eberlin M.N., Augusti R. (2007) Electrospray ionization mass spectrometry fingerprinting of Brazilian artisan cachaca aged in different wood casks. *Journal of Agricultural and Food Chemistry*, **55** (6), 2094–2102.

Gollucke A.P.B., Catharino R.R., de Souza J.C., Eberlin M.N., Tavares D.D. (2009) Evolution of major phenolic components and radical scavenging activity of grape juices through concentration process and storage. *Food Chemistry*, **112** (4), 868–873.

Isidorov V.A., Czyzewska U., Jankowska E., Bakier S. (2011) Determination of royal jelly acids in honey. *Food Chemistry*, **124** (1), 387–391.

Izuta H., Chikaraishi Y., Shimazawa M., Mishima S., Hara H. (2009) 10-Hydroxy-2-decenoic acid, a major fatty acid from royal jelly, inhibits VEGF-induced angiogenesis in human umbilical vein endothelial cells. *Evidence-Based Complementary and Alternative Medicine*, **6** (4), 489–494.

Jiang Y., Cole R.B. (2005) Oligosaccharide Analysis Using Anion Attachment in Negative Mode Electrospray Mass Spectrometry. *Journal of the American Society for Mass Spectrometry*, **16** (1), 60–70.

Kamakura M., Mitani N., Fukuda T., Fukushima M. (2001) Antifatigue effect of fresh royal jelly in mice. *Journal of Nutritional Science and Vitaminology*, **47** (6), 394–401.

Karacal F., Aral F. (2008) Effect of the royal jelly on sperm quality in mice. *Indian Veterinary Journal*, **85** (3), 331–332.

Kohno K., Okamoto I., Sano O., Arai N., Iwaki K., Ikeda M., Kurimoto M. (2004) Royal jelly inhibits the production of proinflammatory cytokines by activated macrophages. *Bioscience Biotechnology and Biochemistry*, **68** (1), 138–145.

Mauri P., Pietta P. (2000) Electrospray characterization of selected medicinal plant extracts. *Journal of Pharmaceutical and Biomedical Analysis*, **23** (1), 61–68.

Moller J.K.S., Adamsen C.E., Catharino R.R., Skibsted L.H., Eberlin M.N. (2007) Mass spectrometric evidence for a zinc-porphyrin complex as the red pigment in dry-cured Iberian and Parma ham. *Meat Science*, **75** (2), 203–210.

Nakaya M., Onda H., Sasaki K., Yukiyoshi A., Tachibana H., Yamada K. (2007) Effect of royal jelly on bisphenol A-induced proliferation of human breast cancer cells. *Bioscience Biotechnology and Biochemistry*, **71** (1), 253–255.

Noda N., Umebayashi K., Nakatani T., Miyahara K., Ishiyama K. (2005) Isolation and characterization of some hydroxy fatty and phosphoric acid esters of 10-hydroxy-2-decenoic acid from the royal jelly of honeybees (Apis mellifera). *Lipids*, **40** (8), 833–838.

Okamoto I., Taniguchi Y., Kunikata T., Kohno K., Iwaki K., Ikeda M., Kurimoto M. (2003) Major royal jelly protein 3 modulates immune responses in vitro and in vivo. *Life Sciences*, **73** (16), 2029–2045.

Olimpia P., Mărghitas L.A., Dezmirean D.S., Oana Lelia M., Laura L. (2008) A characterization about physical-chemical composition of royal jelly. *Bulletin UASVM Animal Science and Biotechnologies*, **65** (1–2), 244–248.

Roesler R., Catharino R.R., Malta L.G., Eberlin M.N., Pastore G. (2007) Antioxidant activity of Annona crassiflora: characterization of major components by electrospray Ionization mass spectrometry. *Food Chemistry*, **104** (3), 1048–1054.

Santos L.S., Catharino R.R., Aguiar C.L., Tsai S.M., Eberlin M.N. (2006) Chemotaxonomic markers of organic, natural, and genetically modified soybeans detected by direct infusion electrospray ionization mass spectrometry. *Journal of Radioanalytical and Nuclear Chemistry*, **269** (2), 505–509.

Takaki-Doi S., Hashimoto K., Yamamura M., Kamei C. (2009) Antihypertensive activities of royal jelly protein hydrolysate and its fractions in spontaneously hypertensive rats. *Acta Medica Okayama*, **63** (1), 57–64.

Viuda-Martos M., Ruiz-Navajas Y., Fernandez-Lopez J., Perez-Alvarez J.A. (2008) Functional properties of honey, propolis, and royal jelly. *Journal of Food Science*, **73** (9), R117–R124.