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Characterization of Royal Jelly by Electrospray Ionization Mass Spectrometry Fingerprinting

Eduardo Morgado Schmidt[1], Ildenize Barbosa da Silva Cunha[1,2], Marcos Nogueira Eberlin[1] and Alexandra Christine Helena Frankland Sawaya[2*]

[1]*Thomson Mass Spectrometry Laboratory, UNICAMP, Campinas, SP, Brazil*
[2]*Department of Plant Biology, Institute of Biology, University of Campinas, SP, Brazil*

Abstract

Royal jelly is an apicultural product, fed to the queen bee and consumed by humans as a health food and traditional medicine; hence its beneficial effects on human health have been the subject of several studies. Since royal jelly is obtained in small amounts and reaches a high market price, it is often adultered with other cheaper substances. since it is prone to degradation at room temperature, lyophilization is used to prolong its shelf life. Due to the complex composition of royal jelly, several different parameters need to be evaluated to determine of freshness, purity and quality; consuming an appreciable amount of sample and time. In this work, electrospray ionization mass spectrometry (ESI-MS) fingerprinting was carried out in the negative ion mode on a high resolution FT-ICR mass spectrometer; marker compounds were identified by comparison of their high resolution mass with data from literature. ESI-MS fingerprinting was capable of characterizing natural, lyophilized, degraded and adultered samples of royal jelly, and indicate marker compounds for each set of samples, with the aid of Principal Component Analysis. The complete analysis, from a simple extraction procedure to ESI (-)-MS fingerprinting, takes only a few minutes and consumes only 50 mg per sample. Therefore, large numbers of samples can be quickly evaluated for purity and freshness in a single-shot approach, while using small amounts of royal jelly per analysis.

Keywords: Electrospray ionization mass spectrometry fingerprinting; ESI-MS fingerprinting; Royal jelly; Honeybees

Introduction

You are what you eat; and honeybees seem to know this principle well, hence to make a queen bee they feed larvae with royal jelly (RJ). Since the queen bee is fertile, larger and lives longer than most (*Apis mellifera*) honeybees, this has led humans to consume RJ in traditional medicine and as a health food. The beneficial effect of RJ on human health is still under debate and investigation [1]. RJ has a complex composition and recent studies have identified lists of volatile and polar components [2]; but it is generally accepted that RJ is composed of approximately 60% water, proteins (42-41% of dry matter), carbohydrates (30% of dry matter), lipids (8-19% of dry matter) and small amounts of minerals, polyphenols and vitamins [3]. Lyophilized RJ is considered to have less than 5% water and to preserve the same proportions of proteins, lipids and carbohydrates in terms of dry mass [3]. An important lipid, 10-hydroxydecenoic acid (10-HDA) is considered a key marker since it appears to be unique to RJ [4], possessing diverse pharmacological activities [2]. A recent article reviewed the present knowledge on the composition and biological activities of RJ [5].

Commercial RJ is produced by grafting young larvae into artificial queen cells, and harvesting this material in one to three days. The composition varies slightly between RJ harvested after 24, 48 or 72 h [1]. Since RJ is obtained in much smaller amounts, as compared to most apicultural products, and reaches a high market price, it has been a major target for adulteration. In an evaluation of the physicochemical properties of RJ by different methods, the addition of substantial amounts of yoghurt, egg white, and/or corn starch was detected [6]. The possibility of contamination of RJ with toxic compounds, such as melamine, has also been explored [7].

Natural RJ is usually stored at -18°C or at 4°C; however lyophilized RJ is considered to be more stable and is usually stored at room temperature and sold in the form of capsules [8]. Lyophilization is considered to be able to maintain the characteristics of RJ since it does not entail heating or the addition of chemical components. However, one of the few studies that evaluated the modifications in RJ composition that may occur due to lyophilization [8] showed that lyophilized RJ was more prone to Maillard reactions than natural RJ and that furosine levels were consequently higher.

Proteins are important components in RJ, but are not good indicators of freshness since a study showed that the amino acid composition of RJ failed to vary linearly with storage time [9]. Other parameters have also been proposed for RJ freshness which includes glucose oxidase enzyme activity and furosine contents [3]. The variation of 10-HDA contents of natural (not lyophilized) samples of RJ maintained at -18°C, 4°C and at room temperature for three months was evaluated but not deemed a good parameter for freshness, since the degradation rate was low even at room temperature, [4] Other indirect parameters such as viscosity, color, sugar content and the presence of specific proteins have also been proposed [10].

Several countries, including Switzerland, Bulgaria and Brazil [3] have defined national standards for RJ. France imports most of its RJ from China, and the main parameters for quality evaluation are moisture, protein and 10-HDA contents [11]. The type of sugar found was indicative of bees being fed on hydrolyzed starch instead of flower nectar, but it was not clear if this affected RJ quality. Fourier transform infrared spectroscopy was proposed as a method to evaluate the protein degradation of RJ during storage [12]. Although RJ is generally considered fresh if under 3 months old, the study showed that after 7 weeks at 4°C or up to 21 weeks at -20°C RJ presented some modifications, but remained stable at room temperature for up to 3 days.

***Corresponding author:** Alexandra Christine Helena Frankland Sawaya, Department of Plant Biology, Institute of Biology, University of Campinas, SP, Brazil, Tel: +55 19 3521 3073; E-mail: achfsawa@unicamp.br

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In fact due to its complex composition, several different parameters need to be evaluated to determine with confidence the freshness, purity and quality of RJ. This task is however quite time and sample consuming, especially as RJ is produced in relatively small amounts and is time and temperature sensitive. These limitations pose a serious challenge for proper RJ quality control. We note however that direct infusion mass spectrometry fingerprinting may offer a solution for RJ quality control since it can be performed with great speed and simplicity, consuming very small amounts of material. Due to its ability to characterize samples at the molecular level, mass spectrometry fingerprinting could provide selective and detailed determination and temporal monitoring of the changing chemical composition of RJ samples. Electrospray ionization (ESI) is effective for compounds of high and medium polarity, and could therefore monitor the peptide, sugar and fatty acid composition of RJ samples. ESI-MS fingerprints have already been used for quality control, adulteration and degradation monitoring of a great variety of complex food and beverage samples [13-15] as well as other apicultural products such as propolis [16,17].

Ultrahigh resolution and mass accuracy mass spectrometers, such as those employing Fourier transform ion cyclotron resonance (FT-ICR) analyzers; have been successfully applied to metabolomics investigations for years. The FTMS approach allows many ion masses to be determined simultaneously and efficiently, with less noise [18-20]. FT-ICR-MS provides high mass accuracy with mass error around 1 ppm, which combined with a soft ionization as electrospray ionization (ESI), enables the determination of molecular formulas from mass measurements only [18]. High resolution and accuracy MS measurements such as those available via FT-ICR-MS permit the determination of the molecular formula of detected components in RJ, as well as sample profiles. This is especially important when studying natural products whose composition has not been fully determined. For the analysis of unknown compounds, the use of FT-ICR-MS allows for the identification of molecular formulas of these ions without the use of standards. After the identification of the marker compounds, other mass spectrometers, such as TOFs and quadrupoles, could be used for the same purpose.

This study has therefore evaluated the applicability of the ESI-MS fingerprinting method, via the evaluation of sample profiles and the detection of typical markers, for RJ typification, adulteration and degradation monitoring as applied to natural and lyophilized samples.

Materials and Methods

Materials

Five samples of natural RJ were obtained from local supermarkets and apicultural product suppliers. For one brand, capsules containing lyophilized RJ were also obtained. For the other four samples an aliquot was lyophilized in our laboratory. These samples present an overview of the products found in local markets. Before analysis, lyophilized samples were re-hydrated. An aliquot of 50 mg of each sample was then dissolved in 1.5 mL of methanol/water 1:1. A volume of 0.5 mL of this solution was transferred to a vial of 1 mL and diluted with 0.5 mL of methanol containing 0.1% ammonium hydroxide for negative ion mode ESI-MS analysis. Samples were extracted and analyzed in triplicate.

Preparation of thermal degradation: For the thermal degradation test, both natural and lyophilized samples of RJ were kept at a temperature of 37°C for 3 days. For the adulteration tests, 30% (v/v) of milk based products (yoghurt or condensed milk) were added to the natural sample of RJ; 30% (m/m) starch powder was added to the lyophilized sample. These samples were then extracted and analyzed as above.

Mass spectrometry and ion identification: Electrospray ionization mass spectra (ESI-MS) fingerprinting was performed using a quadrupole linear trap Fourier transform 7.2T LTQ FT Ultra mass spectrometer (Thermo Scientific, Bremen, Germany) equipped with a chip-based direct infusion Nano electrospray ionization source (Nanomate-Advion Biosciences, Ithaca, NY, USA) operating in the negative ion mode under the follow conditions: a 200 nL/min flow rate, 0.3 psi backing pressure, and 1.5 to 2.0 kV electrospray voltages during 120 s, controlled by Chip Soft software (version 8.1.0, Advion Biosciences). Mass resolution was fixed at 100,000 at *m/z* 400. Data were obtained as transient files (scans recorded in the time domain) and acquisition was performed along the 100-1000 *m/z* range by the Xcalibur 2.0 software.

Identification of the ions was achieved by comparing the high resolution *m/z* values obtained with a library of compounds based on literature search and standards. We considered a match between the experimental *m/z* value and the theoretical *m/z* value from our library when the mass error was <1.0 ppm. Isotopologue distribution pattern of the ions identified was also considered with the proposed chemical formula (Table 1).

Multivariate analysis of data: The *m/z* values and relative abundances for the fifty most abundant ions from each sample were exported from the Xcalibur software and uploaded into the STATISTICA 7 software for multivariate analyses. Matrices, where each line represents a sample and each column a variable, were generated and submitted to Principal Component Analysis (PCA).

Results

Fingerprint method for royal jelly

Although mass spectra were initially acquired with ESI in both positive and negative ion modes, only the negative ion mode (ESI (-)-MS) data were clearly grouped by the PCA, and was therefore selected as the best fingerprinting method for RJ.

Natural and lyophilized samples

Differences between the natural and lyophilized samples could not be easily visualized from the ESI (-)-MS fingerprints (Figure 1A and 1B), but the PCA analysis clearly divided the samples in two quite distinct groups of natural (N) and lyophilized (L) samples, with over 80% of the variability accounted for in the graph of PC1 × PC2 × PC3 (Figure 2). The major marker ion for the natural samples was that of *m/z* 229 which from its accurate *m/z* was identified as deprotonated 10-acetoxydecanoic acid ($C_{12}H_{22}O_4$). The spectra of the lyophilized samples presented a higher number of marker ions, reflecting a higher degree of variability in their composition induced by the lyophilization process. The major marker ions of the lyophilized RJ samples, were a set of deprotonated acids: 8-hydroxy-octanoic acid (*m/z* 159); 10-hydroxy-2-decenoic acid (*m/z* 185); 9,10-dihydroxy-2E-decenoic acid (*m/z* 201); 3,10-dihydroxidecanoic acid (*m/z* 203); pantothenic acid or vitamin B5 (*m/z* 218) and 3,11-dihydroxydodecanoic acid (*m/z* 231). These ions were also detected in the spectra of the natural RJ samples and are consistent with the knowledge that RJ displays indeed a series of C8 to C10 fatty acids. A major and characteristic difference was the ESI (-)-MS detection of acetylated fatty acids only in the natural RJ samples, suggesting that lysis occur to some extent during RJ lyophilization.

Compounds	Formula	Theoretical *m/z*	Experimental *m/z*	Error (ppm)
8-hydroxy-octanoic acid	$C_8H_{15}O_3$	159.10267	159.10282	0.94
10-hydroxy-2-decenoic acid	$C_{10}H_{17}O_3$	185.11832	185.11849	0.91
3-Hydroxydecanoic acid	$C_{10}H_{19}O_3$	187.13397	187.13414	0.89
9,10-dihydroxy-2-decenoic acid	$C_{10}H_{17}O_4$	201.11323	201.11358	0.97
3,10-dihydroxidecanoic acid	$C_{10}H_{19}O_4$	203.12888	203.12901	0.95
10-acetoxydecanoic acid	$C_{12}H_{21}O_4$	229.14453	229.14473	0.94
10,11-dihydroxydodecanoic acid	$C_{12}H_{23}O_4$	231.16018	231.16052	0.93
Dissacharide*	$C_{12}H_{21}O_{11}$	341.10916	341.10894	0.64
Starch*	$C_{16}H_{29}O_9$	365.18171	365.18193	0.61
Dissacharide + Cl*	$C_{12}H_{22}O_{11}{}^{35}Cl$	377.08561	377.08561	0.90

Table 1: Compounds identified in the methanolic extracts of royal jelly analyzed by ESI (-) FT-ICR-MS. *The marker ions for starch and milk-based products used to adulterate RJ.

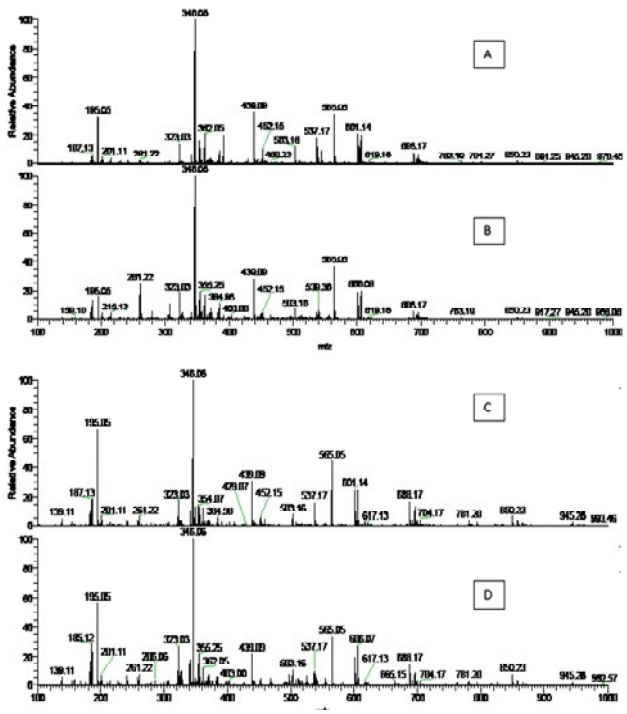

Figure 1: ESI(-)-FTMS of methanolic solutions of RJ samples: A: Fresh natural; B: Lyophilized; C: Natural subjected to thermal degradation; D: Lyophilized subjected to thermal degradation.

Degradation monitoring

For degradation monitoring, both natural and lyophilized RJ samples were kept at a temperature of 37°C for 7 days, then extracted and analyzed by ESI(-)-MS. Again, the differences in the fingerprints are not easily visualized (Figure 1C and 1D) but principal component analysis placed the temperature treated samples (NE and LE) clearly on the left side of the PCA (Figure 3A) and the original natural and lyophilized samples (L and N) were grouped together on the right.

Adulteration monitoring

For adulteration monitoring, samples of RJ mixed with starch and milk-based products such as yoghurt and condensed milk were analyzed and quite distinct ESI-MS fingerprints (Figure 4) were observed, and by PCA (Figure 3B). Starch was added to the lyophilized RJ to simulate a possible form of adulteration, due to similarity in color and consistency. The major marker ion for starch was found to be that of *m/z* 365. The milk-based products are also similar in color and texture to natural RJ, and the major marker ions for these products were the ions of *m/z* 341 and *m/z* 377, corresponding to the deprotonated molecule and chlorine adducts of disaccharides such as lactose and sucrose. A unique trend for adulteration is related to the characteristic ESI(-)-MS markers of *m/z* 195, 346 and 565, which stand out in all the pure RJ fingerprints, but are much less abundant in the adulterated samples. In a sample of natural RJ adulterated with starch, condensed milk and yoghurt, the marker ions of both types of adulteration could still be seen in the ESI(-)-MS fingerprint, but as much less abundant ions.

Discussion

A major and characteristic difference between natural and lyophilized samples was the ESI (-)-MS detection of acetylated fatty acids only in the natural RJ samples, suggesting that lysis occurs to some extent during RJ lyophilization.

The question of whether lyophilization protects RJ from thermal degradation and extends the shelf life of this delicate product was already raised by Messia et al. [8]. These authors found that lyophilized RJ was more susceptible to reactions between sugars and proteins (Maillard reactions). Their results as well as the present ESI(-)-MS data indicate therefore that changes in composition occur during lyophilization and that similar degradation is observed for both samples.

The ESI(-)-MS composition revealed that natural and lyophilized RJ samples kept at 37°C for 7 days were essentially similar, showing that lyophilization of the RJ samples did not protect them from thermal degradation. In fact, ESI (-)-MS data provided evidence for lysis during lyophilization which seems undesirable when the goal is to fully preserve the natural properties of RJ.

RJ adulteration has been evaluated via the physicochemical characteristics of natural and adulterated RJ, and a few general parameters such as moisture, lipid and protein contents have been proposed as indicators for purity [6]. These parameters may however reflect differences between harvesting time and geographical origin of the samples [1] rather than intentional adulteration, and need several laborious analyses to be evaluated. In the present study we have shown that ESI-MS fingerprinting indicated that the sample had been adultered, as well as the type(s) of adulterant added, in a single analysis.

The ultrahigh resolution and accuracy of the Fourier transform ion cyclotron resonance (FT-ICR) analyzer used, allowed us to profile the samples and identify the main chemical markers for natural and lyophilized RJ, as well as for common adulterants and thermally degraded samples, using exclusively mass spectrometry. Neither chromatographic separation nor comparison with standards was needed, which represents a breakthrough in the field of natural products analysis. The markers identified in this study may now be used for routine quality control studies, using less accurate mass spectrometers.

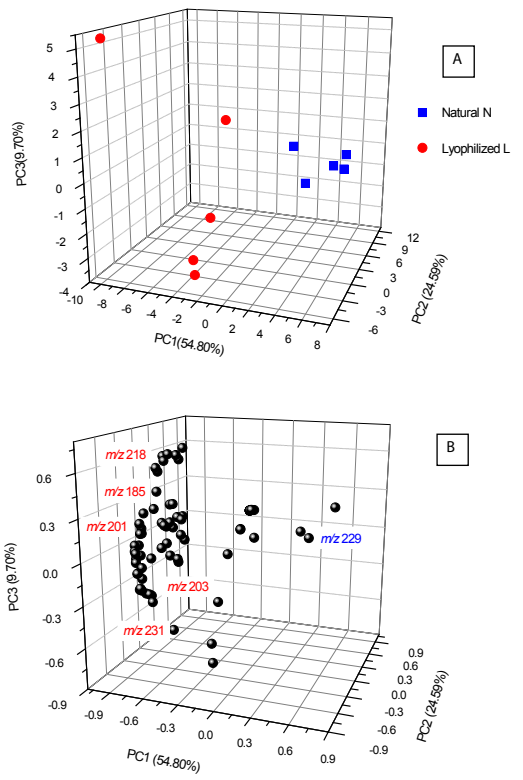

Figure 2: PCA (PC1 × PC2 × PC3) of the ESI(-)-FTMS fingerprints of the (N) natural and (L) lyophilized RJ samples. A: Scores; B: Loadings.

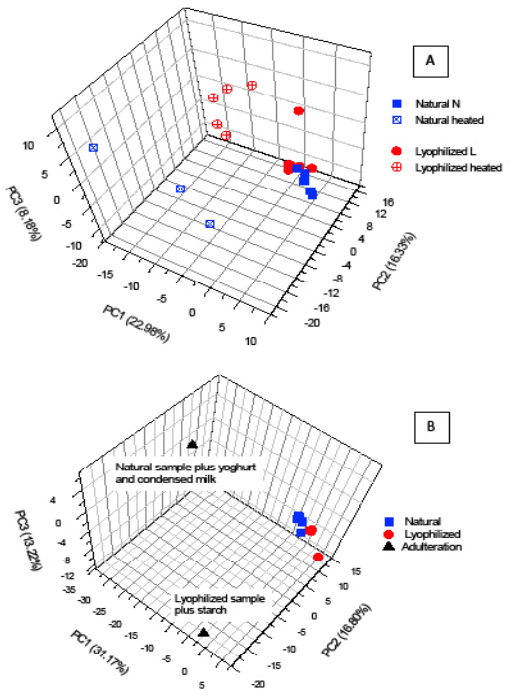

Figure 3: PCA (PC1 × PC2 × PC3) of the ESI(-)-FTMS fingerprints of: A: Natural, lyophilized RJ, lyophilized heated samples and natural heated samples; B: Natural, lyophilized RJ, lyophilized sample plus starch and natural RJ containing yoghurt and condensed milk.

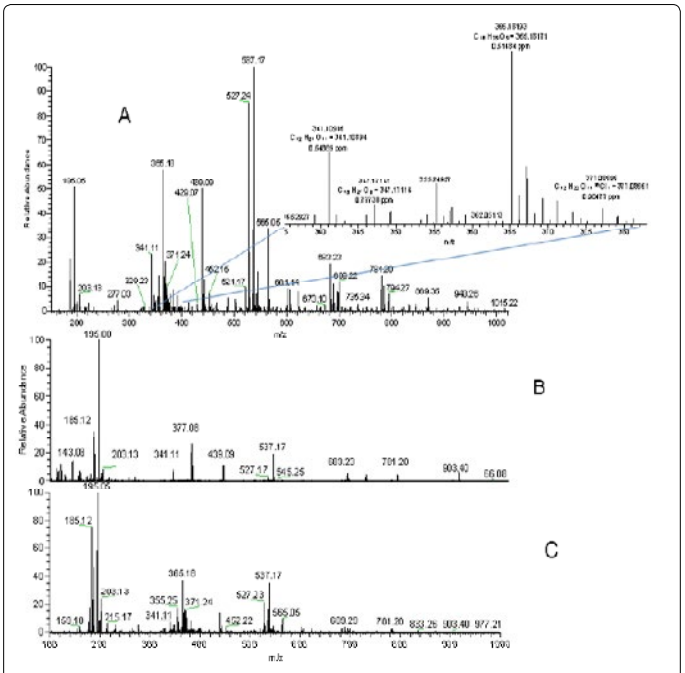

Figure 4: ESI(-)-FTMS of methanolic solutions of adulterated RJ samples: A: Natural plus 30% of a mixture of starch, yoghurt and condensed milk; B: 30% yogurt; C: Lyophilized plus 30% starch. The expansion in A shows the high accuracy *m/z* values and proposed formulas for 3 major marker ions of *m/z* 341, 365 and 377.

ESI(-)-MS fingerprinting, particularly when analyzed via PCA, provides therefore a fast, simple and effective method able to typify RJ samples and to monitor if they are fresh or lyophilized, as well as, adulteration and thermal degradation at the molecular level via characteristic profiles of chemical markers. The major marker ion for the natural samples was that of *m/z* 229 (deprotonated 10-acetoxydecanoic acid) and for the lyophilized RJ samples, major marker ions included 10-hydroxy-2-decenoic acid (*m/z* 185). The marker ions for sugar, milk and starch addition were also identified. These markers ions may be used for routine quality control studies. The complete analysis, from a simple extraction procedure to ESI (-)-MS fingerprinting, takes only a few minutes and consumes only 50 mg per sample. Therefore, large numbers of samples can be quickly evaluated for purity and freshness in a single-shot approach, while using small amounts of RJ per analysis.

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References

1. Zheng HQ, Hu FL, Dietmann V (2011) Changes in composition of royal jelly harvested at different times: consequences for quality standards. Apidologie 42: 39-47.
2. Isidorov VA, Bakier S, Grzech IU (2012) Gas-chromatographic-mass spectrometric investigation of volatile and extractable compounds of crude royal jelly. J Chrom B 885: 109-116.
3. Sabatini AG, Marcazzan GL, Carboni MF, Bogdanov S, Almeida-Muradian LB (2009) Quality and Standardization of Royal Jelly. J ApiProd ApiMed Sci 1: 16-21.
4. Antinelli J, Zeggane S, Davico R, Rognone C, Faucon J, et al. (2003) Evaluation of (E) -10-hydroxydec-2-enoic acid as a freshness parameter for royal jelly. Food Chem 80: 85-89.

5. Ramadan MF, Al-Ghamdi A (2012) Bioactive compounds and health-promoting properties of royal jelly: A review**.** J Functional Foods 4: 39-52.

6. Garcia-Amoedo LH, Almeida-Muradian JB (2007) Physicochemical composition of pure and adulterer royal jelly. Química nova 30: 257-259.

7. Zhou J, Zhao J, Xue X, Chen F, Zhang J, et al. (2010) Determination of melamine in royal jelly and lyophilized powder using ultrasonically assisted extraction by ion-pair HPLC with UV detection. J Sep Sci 33: 167-173.

8. Messia MC, Caboni MF, Marconi E (2005) Storage stability assessment of freeze-dried royal jelly by furosine determination. J Agric Food Chem 53: 4440-4443.

9. Liming W, Jinhui Z, Xiaofeng X, Yi L, Jing Z (2009) Fast determination of 26 amino acids and their content changes in royal jelly during storage using ultra-performance liquid chromatography. J Food Comp Analysis 22: 242-249.

10. Chen C, Chen S (1995) Changes in protein components and storage stability under various conditions. Food Chem 54: 195-200.

11. Daniele G, Casabianca H (2012) Sugar composition of French royal jelly for comparison with commercial and artificial sugar samples. Food Chem 134: 1025-1029.

12. Tarantilis PA, Pappas CS, Alissandrakis E, Harizanis PC, Polissiou MG (2012) Monitoring of royal jelly protein degradation during storage using Fourier transform infrared (FTIR) spectroscopy. J Apicult Res 51: 185-192.

13. Catharino RR, Haddad R, Cabrini LG, Cunha IBS, Sawaya ACHF, et al. (2005) Fast characterization of vegetable oils by electrospray ionization mass spectrometry fingerprinting: classification, adulteration and aging. Anal Chem 77: 7429-7433.

14. Catharino RR, Cunha IBS, Fogaça AO,Facco EMP,Godoy HT, et al. (2006) Characterization of must and wine of six varieties of grapes by direct infusion electrospray ionization mass spectrometry. J Mass Spec 41: 185–190.

15. Sanvido GB, Garcia JS, Corilo Y, Vaz BG, Zacca JJ, et al. (2010) Fast Screening and Secure Confirmation of Milk Powder Adulteration with Maltodextrin via Electrospray Ionization−Mass Spectrometry [ESI(+)−MS] and Selective Enzymatic Hydrolysis. J Agric Food Chem 58: 9407-9412.

16. Sawaya ACHF, Tomazela DM, Cunha IBS, Bankova VS, Marcucci MC, et al. (2004) Electrospray ionization mass spectrometry fingerprinting of propolis. Analyst 129: 739-744.

17. Cunha IBS, Rodrigues MLT, Meurer EC, Bankova VS, Marcucci MC, et al. (2006) Effect of the maceration time on chemical composition of extracts of Brazilian propolis. J Apicult Res 45: 137-144.

18. Aharoni A, de Vos CH, Maliepaard CA, Kruppa G, Bino RJ, et al. (2002) Nontargeted metabolome analysis by use of Fourier transform ion cyclotron mass spectrometry. J Integrat Biol 6: 217-234.

19. Takahashi H, Kai K, Shinbo Y, Tanaka K, Ohta D, et al. (2008) Metabolomics approach for determining growth-specific metabolites based on Fourier transform ion cyclotron resonance mass spectrometry. Anal Bioanal Chem 391: 2769-82.

20. Cuadros-Inostroza A, Giavalisco P, Hummel J, Eckardt A, Willmitzer L, et al. (2010) Discrimination of Wine Attributes by Metabolome Analysis. Anal Chem 82: 3573–3580.