Metabolic fingerprinting of royal jelly: characterization and proof of authenticity

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Abstract
Introduction Metabolic fingerprint is a high-throughput screening to provide a sample classification. Objectives Herein, we describe a metabolic fingerprint 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.

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., 2003).
et al., 2007), biodiesel (Catharino et al., 2007) and micotoxins (Catharino et al., 2005b).

Herein, we report metabolic fingerprint 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% NH₄OH 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
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-2E-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 (CH₂COH), 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 C₁₃H₂₄O₂ and C₁₁H₂₂O₃ 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 concentration in powdered milk, for example, the ion m/z 377 refers to chloride dissacharides, [cellobiose + 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 1](https://example.com/figure1.png)

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

![Figure 2](image1.png)

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

![Figure 3](image2.png)

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

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References


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