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Improving DNA isolation from honey for the botanical origin identification

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ABSTRACT

Honey is a natural product highly consumed due its known association with health benefits. Monofloral honeys are perceived as better quality products, being the most appreciated by consumers, thus attaining higher market values. Therefore efficient tools are needed as alternatives to the classical microscopic analysis presently used for the botanical origin identification of honey. In the present work, the use of DNA-based methods for the botanical species identification of honey is proposed. For this purpose, five DNA extraction methods (the kits NucleoSpin Plant (methods A and B) and DNeasy Plant Mini Kit, and the in-house CTAB-based and Wizard methods) combined with three different sample pre-treatments were applied to four honey samples (3 monofloral honeys of *Calluna vulgaris*, *Lavandula* spp. and *Eucalyptus* spp. and one multifloral honey). The 15 DNA extraction protocols were compared in terms of DNA integrity, yield and purity, as well as capacity of amplification targeting universal and *adh1* specific genes of *C. vulgaris*. The results demonstrated the superior efficacy of the Wizard method in terms of DNA quality and amplification capacity, when combined with the sample preparation treatment with a mechanical disruption step of pollen to improve DNA yield. Although with considerable lower DNA yields, the CTAB and DNeasy methods were also successful because both were able to clearly amplify heather DNA from the monofloral heather honey.

1. Introduction

In the last years, the increase of population concern with health and well-being has led to a growing demand for natural food products. In particular, the rising interest towards foods associated with therapeutic and healing properties has increased their value, making them vulnerable targets to economic frauds. Honey, a natural food produced by *Apis mellifera* bees, is among those products since it is highly consumed for its appreciated taste and also for its potential health benefits and biological properties (Al-Waili & Boni, 2003; Viuda-Martos, Ruiz-Navajas, Fernández-López, & Pérez-Álvarez, 2008; Wang, Andrae, & Engeseth, 2002). Honey can be classified as monofloral, when arising predominantly from a single botanical origin (generally one plant species represents more than 45% of the total pollen content), or multifloral. Because of its refined flavour and taste, monofloral honeys are perceived as better quality products, being the most appreciated by consumers, thus attaining higher market values. Owing to its higher economic value together with the increasing world global trade, monofloral honeys are particularly prone to adulteration through incorrect labelling and fraudulent admixing with cheaper and lower quality honey. In order to protect consumers and promote fair competition among producers, there is a growing need to assess honey's authenticity, in particular to develop methodologies that allow establishing the botanical origin of honey.

Currently, the traditional method used for ascertaining the origin of honey is melissopalynology, which relies on pollen identification by microscopic analysis to determinate the plants visited by the bees during honey's production. However, this method is time consuming, requires the availability of a comprehensive collection of pollen grains and must be performed by experts with adequate skills and experience to identify pollen grains based on its different morphologies. Consequently, in the last years, several other methodologies have been proposed for the determination of

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the botanical origin of honey samples, including the assessment of different chemical parameters such as free amino acids (Hermosı; n, Chicón, & Dolores Cabezudo, 2003), phenolic compounds (Escriche, Kadar, Juan-Borrás, & Domenech, 2014), organic acids (Suárez-Luque, Mato, Huidobro, Simal-Lozano, & Sancho, 2002) and volatile compounds (Cuevas-Glory, Pino, Santiago, & Sauri-Duch, 2007), by means of different analytical instrumentation, including spectroscopic techniques (Arvanitoyannis, Chalhoub, Gotsiou, Lydakis-Simantiris, & Kefalas, 2005; Ruoff et al., 2006). Nevertheless, the chemical composition of honeys with the same botanical origin may be quite different since plant phytochemicals can vary widely (due to edaphoclimatic factors, soil, flower maturity, etc.), making this an unreliable approach for the unequivocal botanical classification of honey (Arvanitoyannis et al., 2005; Kaškonienė & Venskutonis, 2010). Moreover, the need for chemometrics to analyse chemical data often makes it rather difficult to draw reliable conclusions regarding unknown samples. To overcome these drawbacks, the use of DNA markers present in pollen to specifically identify the botanical species of honey is a novel and promising approach. The use of DNA-based methods offer advantages in terms of rapidity, sensitivity and specificity, being suitable for standardisation and thus an alternative to the traditional melissopalynological analysis (Laube et al., 2010). However, to successfully achieve DNA amplification by polymerase chain reaction (PCR), the use of efficient DNA extraction protocols is critical. Ideally, the method of choice should be able to provide high quantity and quality DNA extracts, without potential interfering PCR inhibitors. When dealing with complex matrices having low amounts of target DNA, such as honey, the selection of an adequate DNA extraction method is even more important. Honey is mainly composed of different sugars, but also contains other substances such as organic acids, polyphenols, pigments, enzymes and solid particles as waxes (Codex alimentarius, 2001), which are considered as being PCR inhibitors. Pollen is also present as a characteristic constituent, but at very low levels. For these reasons, sample preparation to isolate pollen particles and eliminate undesirable compounds, such as sugars and flavonoids, are required prior to DNA extraction (Cheng et al., 2007; Laube et al., 2010).

In previous studies, DNA extraction of pollen from honey samples has been reported both by using in-house extraction methods or commercial DNA extraction kits. Cheng et al. (2007) and Waiblinger et al. (2012) both used CTAB-based extraction methods in order to isolate DNA from honey and evaluate the presence of genetically modified organisms. The use of DNeasy Blood and Tissue Kit (Qiagen GmbH) was reported in other works aiming at detecting DNA from different plant species in honey samples (Laube et al., 2010; Valentini, Miquel, & Taberlet, 2010). Recently, Guertler, Eicheldinger, Muschler, Goerlich, and Busch (2014) reported the development of an automated DNA extraction method from pollen in honey and compared its performance with a manual CTAB buffer-based DNA isolation method. Although the automated method proved to be faster than the manual and resulted in higher DNA yield, it requires the use of high-cost instrumentation. However, there is still a scarcity of data concerning comparative analysis of the performance of different DNA extraction methods applied to honey samples.

In the present study, three different sample preparation treatments combined with five different DNA extraction methods were evaluated for the extraction of honey samples of four different botanical origins. The methods were selected taking into consideration previously reported results for DNA extraction from other complex food matrices and included both in-house and commercial kits. The performance of the methods was assessed and compared concerning both the extraction efficiency (DNA quantity and purity) and DNA suitability for amplification.

2. Materials and methods

2.1. Sample preparation

Three different monofloral honeys and one multifloral were used in this work. The monofloral honey samples of heather (*Calluna vulgaris*), lavender (*Lavandula* spp.) and the multifloral honey were acquired from local producers in the northeast region of Portugal (Trás-os-Montes), while the eucalyptus (*Eucalyptus* spp.) honey was obtained from the northwest region of Portugal (Passos de Ferreira). Prior to DNA extraction, each honey was submitted to three different sample preparations, named *a*, *b* and *c*:

- Pre-treatment *a* was performed as described by Cheng et al. (2007) with minor modifications. A sample of 50 g (4×12.5 g) of honey was weighted into four 50 mL Falcon tubes, 2 mL of ultrapure water were added to each tube and the mixtures were stirred. Then, 12 mL of phosphate buffered saline solution (PBS) (136 mM NaCl, 1.4 mM KH_2PO_4, 2.6 mM KCl, 8.09 mM $Na_2HPO_4 \cdot 12H_2O$, pH 7.2) were added to each tube and the mixtures was homogenised for 3 min. After centrifuging at 12,000 *g* for 20 min, the supernatants were discarded. The pellets were re-suspended and combined in 1 mL of ultrapure water and 1 mL of PBS, transferred to a 2 mL tube and centrifuged at 12,000 *g* for 20 min. The supernatant was discarded and the pellet was stored at −20 °C until subsequent DNA extraction.
- Pre-treatment *b* was performed by homogenising 50 g of honey sample in 180 mL of ultrapure water and subsequent distribution of the mixture into four sterile 50 mL centrifugation tubes, which were incubated at 65 °C for 30 min with stirring. The mixture was centrifuged for 30 min at 12,000 *g*, the supernatant was discarded, and each pellet was re-suspended in 400 μL of distilled water, which were further combined into one 2 mL tube. The suspension was placed in an ultrasonic bath (FungiLab SA, Barcelona, Spain) during 2 min. The mixture was stored at −20 °C until subsequent DNA extraction.
- Pre-treatment *c* was performed based on a protocol proposed by Waiblinger et al. (2012) with some modifications. Fifty grams of honey sample were distributed into four sterile 50 mL centrifugation tubes (12.5 g honey per tube), followed by the addition of 40 mL of ultrapure water to each tube, stirring and incubation at 40 °C for 10 min. After centrifugation for 10 min at 11,000 *g*, the supernatants were discarded, each pellet re-suspended in 5 mL of ultrapure water and combined in one 50 mL tube. The suspension was diluted with ultrapure water until a volume of approximately 45 mL and centrifuged for 10 min at 11,000 *g*. The supernatant was discarded, the pellet was re-suspended in approximately 0.5 mL of ultrapure water and transferred to a 2 mL reaction tube containing 7 glass beads (particle size approximately 500 μm). After vortex stirring the suspension for 2 min, the glass beads were removed. The mixture was stored at −20 °C until subsequent DNA extraction.

2.2. DNA extraction

The pre-treated samples with the above mentioned 3 procedures were extracted using five different methods: Nucleospin A, Nucleospin B, DNeasy, Wizard and CTAB.

2.2.1. Nucleospin A and Nucleospin B

The NucleoSpin methods were based on the use of the commercial kit NucleoSpin® Plant II (Macherey−Nagel, Düren, Germany) and performed according to the manufacture instructions with some minor modifications. This kit included two methods of

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