Food Chemistry 214 (2017) 183–191

Contents lists available at ScienceDirect

Food Chemistry

journal homepage: www.elsevier.com/locate/foodchem

Analytical Methods

Rapid identification of the botanical and entomological sources of honey using DNA metabarcoding

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ARTICLE INFO

Article history:
Received 2 March 2016
Received in revised form 7 July 2016
Accepted 11 July 2016
Available online 12 July 2016

Keywords:
Honey
Metabarcoding
Next generation sequencing
Food security
Fraud

ABSTRACT

Honey is generated by various bee species from diverse plants, and because the value of different types of honey varies more than 100-fold, it is a target for fraud. This paper describes a protocol that employs DNA metabarcoding of three gene regions (ITS2, rbcLa, and COI) to provide an inexpensive tool to simultaneously deliver information on the botanical and entomological origins of honey. This method was used to examine seven varieties of honey: light, medium, dark, blended, pasteurized, creamed, and meliponine. Plant and insect sources were identified in five samples, but only the botanical or insect source could be identified in the other two. Two samples were found to be misrepresented. Although this method was generally successful in determining both plant and insect sources, honeys rich in polyphenolic compounds or subject to crystallization were recalcitrant to analysis, so further research is required to combat honey adulteration and mislabeling.

1. Introduction

Derived from pollen and nectar, honey is made by several species of bees aside from the European honey bee, *Apis mellifera* (Jones, 2010). Polyfloral honey is ubiquitous, but monofloral honey can be produced by deploying hives where flowers of a particular plant species are dominant. Due to their rarity, unique flavours, and medicinal properties, monofloral honeys can be very expensive. For example, Manuka honey, which derives from two species of *Leptospermum*, retails at about $100/kg (Ahmed & Othman, 2013) due to its health benefits (Yaghoobi, Kazerouni, & Kazerouni, 2013). The value of honey is also influenced by its insect source; honey from stingless bees (e.g. *Melipona beecheii*) is highly valued, creating an incentive to dilute or substitute with honey from *A. mellifera* (Vit, Medina, & Enriquez, 2004).

While some effort has been made to develop protocols to ascertain the entomological sources of honey (Ramón-Sierra, Ruiz-Ruiz, & de la Luz Ortiz-Vázquez, 2015; Schnell, Fraser, Willerslev, & Gilbert, 2010), most work has focused on identifying its plant origins. Past studies have often relied upon diagnostic phytochemicals (e.g. Cotte et al. (2004), Tosun (2013)) or melissopalynology, the study of pollen in honey (e.g. de Franca Alves and de Assis Ribeiro dos Santos (2014)). Although the latter approach requires considerable expertise and cannot distinguish many plant species (Kaskoniene & Venskutonis, 2010), it is a powerful diagnostic tool, especially when used with other methods (Hawkins et al., 2015). However, melissopalynology is ineffective in cases where low-value honey is filtered to remove its source pollen and spiked with pollen from the 'desired' monofloral.

Although none of the above protocols can reliably determine the plant source of an 'unknown' honey (Kaskoniene & Venskutonis, 2010; Laube et al., 2010), such uncertainty can often be resolved through the genetic analysis of targeted gene regions isolated from honey. Termed metabarcoding, this approach overcomes many of the limitations of other analytical methods (Schnell et al., 2010) and is gaining power because of increased access to high-throughput sequencing platforms (Hawkins et al., 2015; Richardson et al., 2015; Valentini, Miquel, & Taberlet, 2010). Although honey includes pollen, nectar, and bee products, most prior genetic studies have only examined pollen (Hawkins et al., 2015; Richardson et al., 2015; Valentini et al., 2010). While such analysis reveals the major botanical components of honey, full authentication requires analysis of DNA isolated from liquid honey because it can reveal both the source bee and "hidden" floral components in cases where honey has been filtered to remove pollen.

This paper describes a cost-effective protocol to identify the botanical and entomological components of honey using metabarcoding to examine three gene regions. Pollen components are examined using nuclear ITS2, pollen-free plant material with the plastid gene rbcLa, and the source bee species using mitochondrial

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http://dx.doi.org/10.1016/j.foodchem.2016.07.077
0308-8146/

cytochrome *c* oxidase subunit I (COI). ITS2 (~350 bp) was chosen to identify the pollen signature of honey because it can discriminate most plant species (Yao et al., 2010). Additionally, ITS2 is found in all pollen grains, while plastid markers can be absent (Bell, Burgess, Okamoto, Aranda, & Brosi, 2016). A segment (162 bp) of the rbcLa gene was used to detect trace and/or degraded plant DNA in honey (but not necessarily from pollen). Finally, a segment (120 bp) of the COI gene was used to identify the entomological source of honey. After developing a protocol for the authentication of honey, its performance was tested on seven of the most common forms of honey to ascertain its advantages and limitations.

2. Materials and methods

2.1. Honey samples

Seven honey samples were examined (Table 1); six from *A. mellifera* obtained from retail outlets or apiarists in Ontario, and one from *M. beecheii* obtained from a producer in Mexico. The six samples from *A. mellifera* represented the major types of commercial honey. Light-coloured honey is the most common form and was likely the type analyzed in previous studies (e.g. Bruni et al. (2015), Jain, de Jesus, Marchioro, and de Araujo (2013), Valentini et al. (2010)). Three other liquid honeys (medium, dark, and blueberry-flavoured) were analyzed to ascertain if genetic analysis was impeded by higher levels of polyphenols or flavonoids (Pyrzynska & Biesaga, 2009). The last two samples included a liquid and a creamed honey that had undergone pasteurization and controlled-crystallization, respectively. They were included to ascertain if this additional processing affected genetic analysis. Finally, the most valuable honey produced in Mexico and Guatemala, meliponine, was analyzed to confirm that it derived from *M. beecheii* because it is exempt from the oversight imposed on honey producers (Vit et al., 2004) and is thus a target for fraud.

2.2. Preparation of honey for DNA extraction

When viewed from the perspective of DNA analysis, honey includes two components – pollen and liquid. The pollen component contains DNA from the major botanical sources, while the liquid may contain DNA from minor botanical sources and its source insect. DNA was extracted from a homogenized aliquot of liquid or semi-solid honey to examine the liquid component. For pollen analysis, DNA was extracted after concentrating the pollen grains by first heating 10 mL of honey and vortexing it with a 4:1 vol of sterile water at 56 °C. This mixture was centrifuged at 5000*g* for 30 min to pellet the pollen and the supernatant was decanted. The pollen pellet was then re-suspended in 25 mL of sterile 56 °C water and centrifuged at 5000*g* for 15 min. The supernatant was decanted and the pollen pellet was re-suspended in 1 mL of 50% ethanol, transferred to a 2 mL microfuge tube, and centrifuged at 12,000*g* for 15 min. The supernatant was decanted, and the pollen pellet was dried at 56 °C for 45 min. When DNA recovery was compared using three methods (unground, ground with 3 mm tungsten beads, and ground with sand), the first two methods produced DNA concentrations that were just 1% of those from pollen ground with sand (data not shown). Therefore, 100 mg of sterile sand (grains 0.3 mm in diameter) was added to the dried pellet, and the pollen/sand mixture was manually ground with a pestle to pulverize the pollen prior to DNA extraction.

2.3. DNA extraction

DNA was extracted using 400 μL of lysis buffer [700 mM guanidine thiocyanate (Sigma), 30 mM EDTA pH 8.0 (Fisher Scientific), 30 mM Tris-HCl pH 8.0 (Sigma), 0.5% Triton X-100 (Sigma), 5% Tween-20 (Fluka Analytical)] mixed with 2 mg/mL of Proteinase K (Promega) prior to use. This lysis buffer was added to 100 μL of liquid honey or to the ground pollen/sand mixture, followed by incubation for 18 h at 56 °C with gentle shaking.

DNA purification employed the method of Ivanova, Dewaard, and Hebert (2006) with slightly different volumes for the liquid and pollen lysates. The lysate was mixed with two volumes – 1 mL (liquid) or 800 μL (pollen) – of binding mix [3 M guanidine thiocyanate, 10 mM EDTA pH 8.0, 5 mM Tris-HCl pH 6.4, 2% Triton X-100, 50% ethanol] and applied to a silica membrane spin column (Epoch Biolabs), 700 μL at a time. The column was centrifuged at 6000*g* for 2 min after each application. The membrane was washed once with 500 μL (liquid) or 350 μL (pollen) of protein wash buffer [1.56 M guanidine thiocyanate, 5.2 mM EDTA pH 8.0, 2.6 mM Tris-HCl pH 6.4, 1.04% Triton X-100, 70% ethanol] and centrifuged at 6000*g* for 2 min. The column was washed a second time with 750 μL of wash buffer [50 mM NaCl (Fisher Scientific), 0.5 mM EDTA pH 8.0, 10 mM Tris-HCl pH 7.4, 60% ethanol] and centrifuged at 6000*g* for 4 min. The flow-through was discarded and the column was centrifuged at 10,000*g* for 4 min. The silica membrane insert was then transferred to a clean 1.5 mL microfuge tube and dried at 56 °C for 30 min.

To release DNA from the silica membrane, 40 μL (liquid) or 50 μL (pollen) of elution buffer [10 mM Tris-HCl, pH 8.0, prewarmed to 56 °C] was applied directly to the membrane and allowed to incubate at room temperature for 1 min. DNA was eluted from the column via centrifugation at 10,000*g* for 5 min. The pollen DNA was quantified using a Qubit 2.0 fluorometer (Life Technologies) and adjusted to approximately 0.5 ng/μL with elution buffer. The DNA from liquid honey was in such low abundance that it could not be quantified to allow normalization, so it was directly used for PCR.

2.4. PCR amplification for Ion Torrent sequencing

PCR amplicons require adapter sequences to enable their characterization on an Ion Torrent PGM (Life Technologies) and multiplex identifier (MID) tags to associate the sequence reads with a particular honey sample. This is typically accomplished with

Table 1
Types of honey analyzed in this study. All information regarding provenance, production, and advertised botanical and entomological sources are directly or indirectly extracted from labels on the container.

Honey type	Provenance	Produced in	Sold in	Advertised botanical source	Advertised entomological source
Light	Canada	Canada	Canada	Polyfloral	*A. mellifera*
Medium	Australia/Brazil	Australia	Canada	Orange blossom	*A. mellifera*
Dark	Canada	Canada	Canada	Buckwheat	*A. mellifera*
Blended	Canada	Canada	Canada	Polyfloral with blueberry extract	*A. mellifera*
Pasteurized	Canada	Canada	Canada	Polyfloral	*A. mellifera*
Creamed	France	France	Canada	Lavender	*A. mellifera*
Meliponine	Mexico	Mexico	Mexico	Polyfloral	*M. beecheii*