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A novel, direct, reagent-free method for the detection of beeswax adulteration by single-reflection attenuated total reflectance mid-infrared spectroscopy

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ABSTRACT

In this work, a novel, direct, reagent-free method for the detection of beeswax adulteration by paraffin, microcrystalline wax, tallow and stearic acid using single-reflection attenuated total reflectance mid-infrared spectroscopy was developed. The use of the absorbance ratios of $I_{1739\text{ cm}^{-1}}/I_{2852\text{ cm}^{-1}}$, $I_{1714\text{ cm}^{-1}}/I_{2852\text{ cm}^{-1}}$ and $I_{1739\text{ cm}^{-1}}/I_{1714\text{ cm}^{-1}}$ allows a minimum of 5% paraffin/microcrystalline wax and tallow adulteration and 0.5% stearic acid adulteration of beeswax to be detected. The upper and lower critical limits for beeswax authenticity were established from the analysis of virgin beeswax and were validated by independent analysis of real sheet and comb beeswax samples using high-temperature gas chromatography with flame-ionization detection. In addition to its simplicity with respect to sample handling, the amount of sample and the time needed are far less than those required in previously described methods, which are based on chemical analysis and chromatographic techniques. These advantages result in time and cost savings, an increase in the number of samples that can be analyzed, and, most importantly, the detection of the main beeswax adulterants using a single method.

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1. Introduction

Beeswax adulteration causes serious economic and apicultural losses, decreasing honey production and quality as well as decreasing honeybees' wellbeing. The high price of beeswax when compared with other solid fatty products makes beeswax an attractive target for adulteration. The most common adulterants added to beeswax are paraffin, microcrystalline wax, tallow and stearic acid. Unsurprisingly, several studies have been conducted to determine authentication parameters for beeswax, including the use of physicochemical parameters [1–6] and high-temperature gas chromatography with flame-ionization detection (HT-GC/FID) [2,7–14]. All of these methods, which exhibit different detection levels of 10% for paraffin, 2% for stearic acid and 10% for tallow adulteration using the classical methods [6] and 1–4% for each adulterant using the chromatographic methods [5,12,14], can detect the most common adulterants. Nevertheless, the traditional methods are time-consuming, involve intensive sample manipulation and, in some methodologies, involve multiple determinations using different methods [12,13]. To develop a fast method without any sample manipulation for the detection of the most common beeswax adulterants, we explore the use of single-reflection attenuated total reflectance mid-infrared spectroscopy

(ATR–FTIR). The ATR–FTIR technique has some advantages for attaining the proposed goal: it is a rapid, nondestructive method that typically does not require any sample manipulation or chemical preparations [15]. These advantages result in time and cost savings and an increase in the number of samples that can be analyzed. Attenuated total reflectance sampling accessories have been widely used in the development of FTIR methods for the analysis of fats and oils [16–19] because they provide a simple and convenient means of sample handling [15,20]. Solid fats are simply melted onto the surface of an ATR crystal, provided that the crystal is maintained at a temperature greater than the melting point of the fat. When only small amounts of sample are available, the single-reflectance ATR accessories are particularly useful because of the small amount of sample required (< 20 mg) to cover the surface of the ATR crystal.

The purpose of this work was to develop a novel, direct, reagent-free method for the detection of beeswax adulterated with paraffin, microcrystalline wax, tallow and stearic acid by single-reflection ATR–FTIR.

2. Material and methods

2.1. Material and reagents

Beeswax samples were collected in the Portuguese market in 2010/2011. Virgin beeswax samples (31) came directly from the

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bee scales because beekeepers had placed an empty frame in a beehive in their apiaries. Comb beeswax samples were also furnished by beekeepers before the recycling process; the samples comprise old combs and beeswax cappings melted together (49). Foundation beeswax samples were bought locally (41) and were also submitted by beekeepers (4). All samples were stored at room temperature and in darkness until they were analyzed. These samples were directly analyzed without any purification step. Chloroform was supplied by Sigma-Aldrich (St. Louis, MO, USA).

2.2. FT-IR spectrum acquisition and data pre-treatment

The FT-IR spectra of beeswax samples were obtained using a Golden Gate single-reflection diamond ATR system (Specac Limited, England) in a Unicam Research Series spectrometer. The spectra were recorded in absorbance mode from 4000 to 650 cm^{-1} (mid-infrared region) at a resolution of 4 cm^{-1} . Five replicate spectra (128 co-added scans) were collected for each sample. The temperature of the crystal was maintained at 75 °C to allow the analysis of beeswax in its liquid state. At the end of each acquisition, the crystal surface was carefully cleaned with 95% ethanol. A background spectrum was acquired after every five samples. Each replicate was acquired on different days, and the sequence of acquisition on each day was randomly assigned using a random number table. The standard deviation obtained for the replicate analysis of each ratio was pooled and used to calculate the method precision at each wavenumber.

After the spectra were acquired, the noise was removed in the regions 4000–2991 cm^{-1} and 2805–1791 cm^{-1} using the lineout macro of the Winfirst 3.2 software, and the baseline was manually adjusted in the regions 4000–2991 cm^{-1} , 2991–2805 cm^{-1} and 2805–1791 cm^{-1} . After the baselines were adjusted, the spectra were normalized to 1 to remove the effect of the variable purity of the comb beeswax samples.

After the spectra were normalized, the following absorbance ratios were used for the calculation of the quality index developed in this work: $I_{1739 \text{ cm}^{-1}}/I_{2852 \text{ cm}^{-1}}$, $I_{1714 \text{ cm}^{-1}}/I_{2852 \text{ cm}^{-1}}$ and $I_{1739 \text{ cm}^{-1}}/I_{1714 \text{ cm}^{-1}}$. To reduce the effect of noise in these spectral regions, instead of using only the absorbance for one wavenumber, the average absorbance of two neighboring wavenumbers were used: $I_{2852 \text{ cm}^{-1}} = (I_{2848} + I_{2850} + I_{2852} + I_{2854} + I_{2856})/5$; $I_{1739 \text{ cm}^{-1}} = (I_{1735} + I_{1737} + I_{1739} + I_{1741} + I_{1743})/5$ and $I_{1714 \text{ cm}^{-1}} = (I_{1710} + I_{1712} + I_{1714} + I_{1716} + I_{1718})/5$.

2.3. Beeswax adulteration by paraffin, microcrystalline wax, tallow and stearic acid

To determine the performance of the proposed quality index: $I_{1739 \text{ cm}^{-1}}/I_{2852 \text{ cm}^{-1}}$, $I_{1714 \text{ cm}^{-1}}/I_{2852 \text{ cm}^{-1}}$ and $I_{1739 \text{ cm}^{-1}}/I_{1714 \text{ cm}^{-1}}$, and the minimum percentage of adulterants detected by the developed ATR-FTIR method, virgin beeswax samples were separately mixed with increasing amounts of adulterants (0.5%, 1.0%, 1.5%, 2.0%, 2.5%, 5.0%, 10%, 15%, 20%, 25% and 30%) and were subsequently melted and solidified by cooling to room temperature. Five independent replicates were used.

2.4. High-temperature gas chromatography

The hydrocarbon and monoester contents of the beeswax samples were determined by high-temperature gas chromatography using flame ionization detection by the method developed previously [21]. Analyses were performed in a Thermo-Finnigan trace gas chromatograph (USA) equipped with a flame ionization detector and an AS3000 automatic sampler (Thermo-Finnigan). For the beeswax analysis, a 30 m ZB-5 Inferno column (Phenomenex Torrance, USA) with 0.25 mm ID and 0.25 μm film thickness was used. The injector temperature was optimized, and the

optimum temperature was 325 °C. A 1 μL sample was always injected in splitless mode with a 2 min splitless time. The following oven temperature program was used: initial temperature 50 °C, which was held for 3 min, a 50 °C min^{-1} ramp to 180 °C, which was held for 1 min, and then a 3 °C min^{-1} ramp to 390 °C, which was held for 5 min. The carrier gas (He) flow rate was constant at 1 mL min^{-1} . The detector temperature was 400 °C. Hydrogen (20 mL min^{-1}) and synthetic air (200 mL min^{-1}) were used as auxiliary gases for the flame ionization detector. For the sample preparation, approximately 3 mg of beeswax was dissolved in 4 mL of chloroform. The solution was mechanically shaken for 2 min to complete the dissolution of the beeswax.

2.5. Statistical analysis

The statistical significance ($p < 0.05$) of the results obtained for the quality index (i.e., $I_{1739 \text{ cm}^{-1}}/I_{2852 \text{ cm}^{-1}}$, $I_{1714 \text{ cm}^{-1}}/I_{2852 \text{ cm}^{-1}}$ and $I_{1739 \text{ cm}^{-1}}/I_{1714 \text{ cm}^{-1}}$) was evaluated by analysis of variance using the software STATISTICA, version 8.0 (2007) for Windows, from Statsoft. Post-hoc analysis was performed using the Fisher least-squares procedure at $p < 0.05$.

3. Results and discussion

3.1. Spectral features

In Fig. 1(a) the mid-infrared spectra of a virgin beeswax sample acquired at 75 °C are shown. The spectra for the non-adulterated

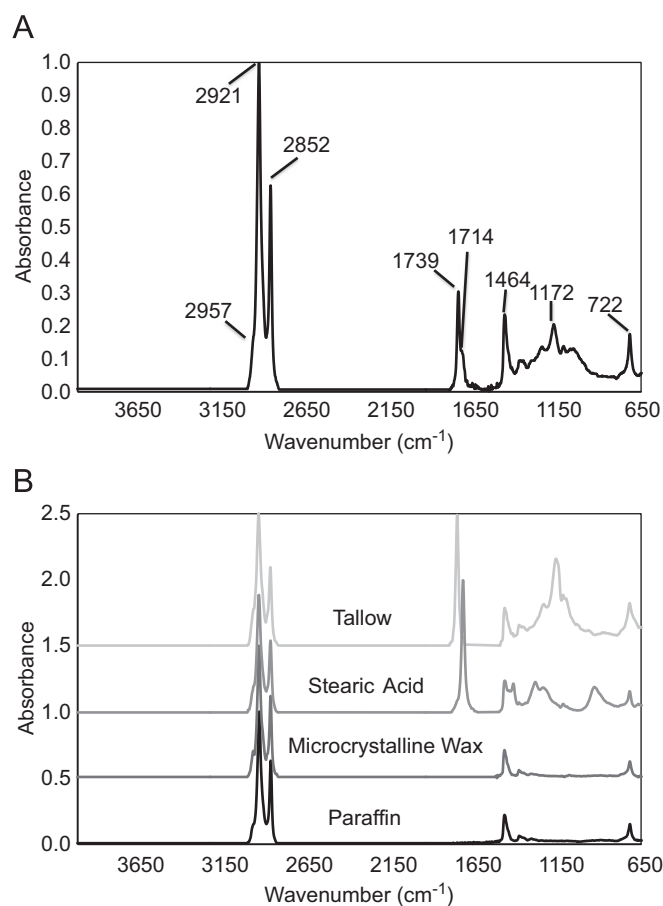


Fig. 1. Infrared spectrum of (A) virgin beeswax and (B) common beeswax adulterants.

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