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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 $I_{1739\ cm^{-1}}/I_{2852\ cm^{-1}}$, $I_{1714\ cm^{-1}}/I_{2852\ cm^{-1}}$ and $I_{1739\ cm^{-1}}/I_{1714\ cm^{-1}}$ 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.

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: $I_{1739\ cm^{-1}}/I_{2852\ cm^{-1}}$, $I_{1714\ cm^{-1}}/I_{2852\ cm^{-1}}$ and $I_{1739\ cm^{-1}}/I_{1714\ cm^{-1}}$. 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: $I_{2852\ cm^{-1}}=(I_{2848}+I_{2850}+I_{2852}+I_{2854}+I_{2856})/5$; $I_{1739\ cm^{-1}}=(I_{1735}+I_{1737}+I_{1739}+I_{1741}+I_{1743})/5$ and $I_{1714\ cm^{-1}}=(I_{1710}+I_{1712}+I_{1714}+I_{1716}+I_{1718})/5$.

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

To determine the performance of the proposed quality index: $I_{1739\ cm^{-1}}/I_{2852\ cm^{-1}}$, $I_{1714\ cm^{-1}}/I_{2852\ cm^{-1}}$ and $I_{1739\ cm^{-1}}/I_{1714\ cm^{-1}}$, 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., $I_{1739\ cm^{-1}}/I_{2852\ cm^{-1}}$, $I_{1714\ cm^{-1}}/I_{2852\ cm^{-1}}$ and $I_{1739\ cm^{-1}}/I_{1714\ cm^{-1}}$) 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

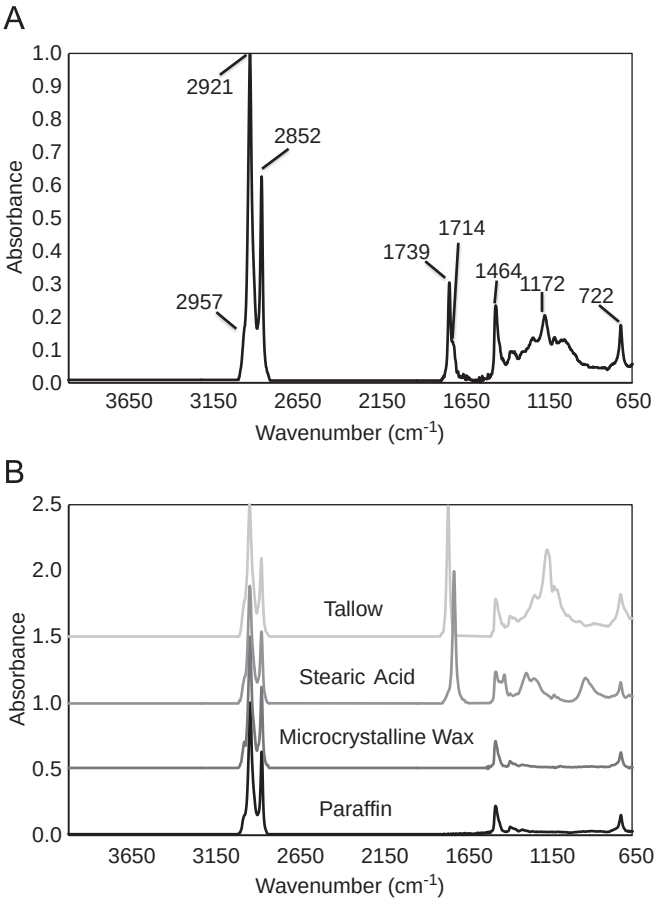

Fig. 1. Infrared spectrum of (A) virgin beeswax and (B) common beeswax adulterants.

comb and sheet beeswax samples were similar to those for the virgin beeswax samples. The spectra obtained for melted beeswax exhibited a higher signal to noise ratio than that acquired using solid beeswax due to better contact between the liquid beeswax and the ATR crystal. Beeswax is a complex chemical mixture of linear hydrocarbons (14%, mainly with odd numbers of carbon atoms), monoesters (35%; predominantly palmitates) and free acids (13%, mainly palmitic acid) [1–9,22]. Because hydrocarbons, monoesters and free fatty acids are the principal beeswax components, the main absorption bands observed in the infrared spectrum are related to these substances. Three vibrations are observed in the 2840–2960 cm^{-1} range. They are assigned to the C–H stretching of methyl (CH_3) and methylene (CH_2) groups [23–25], which are common structural elements of all beeswax components. The vibration at 2957 cm^{-1} (shoulder) is assigned to the asymmetric stretching band of a CH_3 group. The two intense bands at 2921 cm^{-1} and 2852 cm^{-1} are due to CH_2 asymmetric and symmetric stretching vibrations, respectively. The band at 1738 cm^{-1} is assigned to the C=O stretching vibration of the carboxylic groups involved in an ester linkage [23,25,26]. This absorption can be attributed to the monoesters present in beeswax. At 1714 cm^{-1}, a band which is assigned to the C=O stretching vibration of the carboxylic groups of the free carboxylic acids is observed [25,27]; this vibration is attributed to the free fatty acids present in beeswax. The band observed at 1464 cm^{-1} is assigned to CH_2 scissor deformation vibrations [28,29]. In the region from 1290 to 1040 cm^{-1}, a band at 1172 cm^{-1} is observed and is attributed to C=O stretching and C–H bending vibrations [25]. The band at 722 cm^{-1} is assigned to the CH_2 rocking mode [25,29].

Figure 1(b) shows the ATR–FTIR spectrum of the most common beeswax adulterants, i.e., paraffin, microcrystalline wax, tallow and stearic acid. Obvious differences between the infrared spectra of beeswax and those of the adulterants can be observed. For paraffin and microcrystalline wax, as expected, no band is observed in the 1750–1700 cm^{-1} range. The main bands for these two compounds are the vibrations at 2957 cm^{-1} (asymmetric stretching band of a CH_3 group), 2921 cm^{-1} and 2852 cm^{-1}, which are due to CH_2 asymmetric and symmetric stretching vibrations, respectively. Paraffin and microcrystalline wax exhibited similar spectra (Fig. 1b), with the only noticeable difference being the intensities of the peaks at 2957 cm^{-1}. This difference is because microcrystalline wax contains a higher percentage of ramified hydrocarbons in relation to paraffin that is composed of linear hydrocarbons [30].

For tallow, the relative intensity of the band at 1745 cm^{-1}, which is attributed to the C=O stretching vibration of the carboxylic groups involved in the ester bonds of the triglycerides, is significantly greater than that observed for beeswax. Additionally, for stearic acid, the intensity of the band at 1711 cm^{-1}, attributed to the C=O stretching vibration of the carboxylic groups of stearic acid, is significantly greater than that observed for beeswax.

3.2. Feasibility of the ATR–FTIR method for the detection of beeswax adulteration and the limit of detection

To exploit the feasibility of the single-reflection ATR–FTIR method for the detection of paraffin, microcrystalline wax, tallow and stearic acid beeswax adulteration, virgin beeswax samples were mixed with increasing amounts (0.5%, 1.0%, 1.5%, 2.0%, 2.5%, 5.0%, 10%, 15%, 20%, 25%, 30%) of each adulterant. The spectra were acquired, and spectral regions were selected for calculating the discrimination ratios. Based on the comparison of the virgin beeswax spectrum with that of the four adulterants, the following ratios were chosen: $I_{1739\ cm^{-1}}/I_{2852\ cm^{-1}}$, $I_{1714\ cm^{-1}}/I_{2852\ cm^{-1}}$ and $I_{1739\ cm^{-1}}/I_{1714\ cm^{-1}}$. To decrease the effect of noise and to compensate for the fact that the wavenumbers of the C=O stretching vibrations of the carboxylic acid groups in tallow and stearic acid are not exactly the same as those observed for beeswax, the average values around the selected wavenumbers were used. The adulteration of beeswax with paraffin and microcrystalline wax could not be distinguished by ATR–FTIR. Additionally, the ATR–FTIR spectra of paraffins with different melting points, i.e., 42–44 °C, 46–48 °C, 51–53 °C, 56–58 °C, 60–65 °C and 70–80 °C, were similar (results not shown). Therefore, paraffin adulteration was only evaluated using paraffin with a 46–48 °C melting point, which is representative of both paraffin (irrespective of the melting point) and the microcrystalline wax adulteration of beeswax.

The adulteration of beeswax with paraffin results in a significant change in the $I_{1739\ cm^{-1}}/I_{2852\ cm^{-1}}$ ($F=104.6$, $p<0.0001$) and $I_{1714\ cm^{-1}}/I_{2852\ cm^{-1}}$ ($F=18.95$, $p<0.0001$) ratios but not in the $I_{1739\ cm^{-1}}/I_{1714\ cm^{-1}}$ ratio ($F=1.001$, $p<0.4594$; Fig. 2). Post-hoc analysis shows that, for the $I_{1739\ cm^{-1}}/I_{2852\ cm^{-1}}$ ratio, the addition of 5% paraffin is the first concentration where a significant change is observed (mean difference -0.01721 ± 0.007087; $p<0{,}019$) in this ratio in relation to the original sample. A significant linear decrease in this ratio in the range of 5%–30% paraffin adulteration ($I_{1739\ cm^{-1}}/I_{2852\ cm^{-1}}=-0.004934\ C_{Paraffin}+0.4787$, $r=0.960$, $F=326.4$, $p<0.0001$) is observed. For the $I_{1739\ cm^{-1}}/I_{2852\ cm^{-1}}$ ratio, post-hoc analysis shows that the first concentration of paraffin where a significant difference is observed is 10% (mean difference $-0.01677\pm0{,}00621$; $p<0{,}014$). Additionally, in the 10%–30% paraffin adulteration, a significant linear decrease in this ratio ($I_{1714\ cm^{-1}}/I_{2852\ cm^{-1}}=-0.001839\ C_{Paraffin}+0.1756$, $r=0.914$; $F=131.1$, $p<0.0001$) is observed. These results show that the paraffin adulteration of beeswax with a minimum amount of 5% can be determined using the $I_{1739\ cm^{-1}}/I_{2852\ cm^{-1}}$ ratio.

The results obtained for the tallow adulteration of beeswax are presented in Fig. 2. The adulteration of beeswax with tallow results in a significant change in the $I_{1739\ cm^{-1}}/I_{2852\ cm^{-1}}$ ($F=187.2$, $p<0.0001$), $I_{1714\ cm^{-1}}/I_{2852\ cm^{-1}}$ ($F=11.49$, $p<0.0001$) and $I_{1739\ cm^{-1}}/I_{1714\ cm^{-1}}$ ($F=125.1$, $p<0.0001$) ratios. Post-hoc analysis shows that, for the $I_{1739\ cm^{-1}}/I_{2852\ cm^{-1}}$ ratio, the addition of 5% tallow is the first concentration where a significant change (mean difference 0.01232 ± 0.00535; $p<0{,}026$) in relation to the original sample is observed. For beeswax adulterated with 5%–30% tallow, a significant linear increase in the $I_{1739\ cm^{-1}}/I_{2852\ cm^{-1}}$ ratio ($I_{1739\ cm^{-1}}/I_{2852\ cm^{-1}}=0.005316\ C_{tallow}+0.4419$, $r=0.984$, $F=834.0$, $p<0.0001$) is observed. For the $I_{1714\ cm^{-1}}/I_{2852\ cm^{-1}}$ ratio, post-hoc analysis shows that the first concentration of cow tallow where a significant difference is observed is also 5% (mean difference -0.01161 ± 0.00333; $p<0{,}0011$). At greater concentrations, a significant linear decrease in this ratio ($I_{1739\ cm^{-1}}/I_{2852\ cm^{-1}}=-0.0006028\ C_{Tallow}+0.1746$, $r=0.663$; $F=22.0$, $p<0.0001$) is observed. For the $I_{1739\ cm^{-1}}/I_{1714\ cm^{-1}}$ ratio, post-hoc analysis shows that the first concentration of cow tallow where a significant difference is observed is also 5% (mean difference 0.2478 ± 0.0567; $p<0{,}000066$). A significant linear increase in this ratio ($I_{1739\ cm^{-1}}/I_{1714\ cm^{-1}}=0.04481\ C_{Tallow}+2.492$, $r=0.960$; $F=330.2$, $p<0.0001$) is observed with increasing amounts of tallow added to the beeswax. Based on the obtained results, tallow adulteration of beeswax at concentrations greater than 5% can be determined using any of the proposed ratios; however, the $I_{1739\ cm^{-1}}/I_{1714\ cm^{-1}}$ ratio shows the best performance because this ratio exhibits a greater variation with the unit percentage of added adulterant.

The adulteration of beeswax with stearic acid (Fig. 2) results in a significant change in the $I_{1739\ cm^{-1}}/I_{2852\ cm^{-1}}$ ($F=30.54$, $p<0.0001$), $I_{1714\ cm^{-1}}/I_{2852\ cm^{-1}}$ ($F=2180$, $p<0.0001$) and $I_{1739\ cm^{-1}}/I_{1714\ cm^{-1}}$ ($F=1185$, $p<0.0001$) ratios. Post-hoc analysis shows that, for the $I_{1739\ cm^{-1}}/I_{2852\ cm^{-1}}$ ratio, the addition of 10% stearic acid is the first concentration where a significant change (mean difference

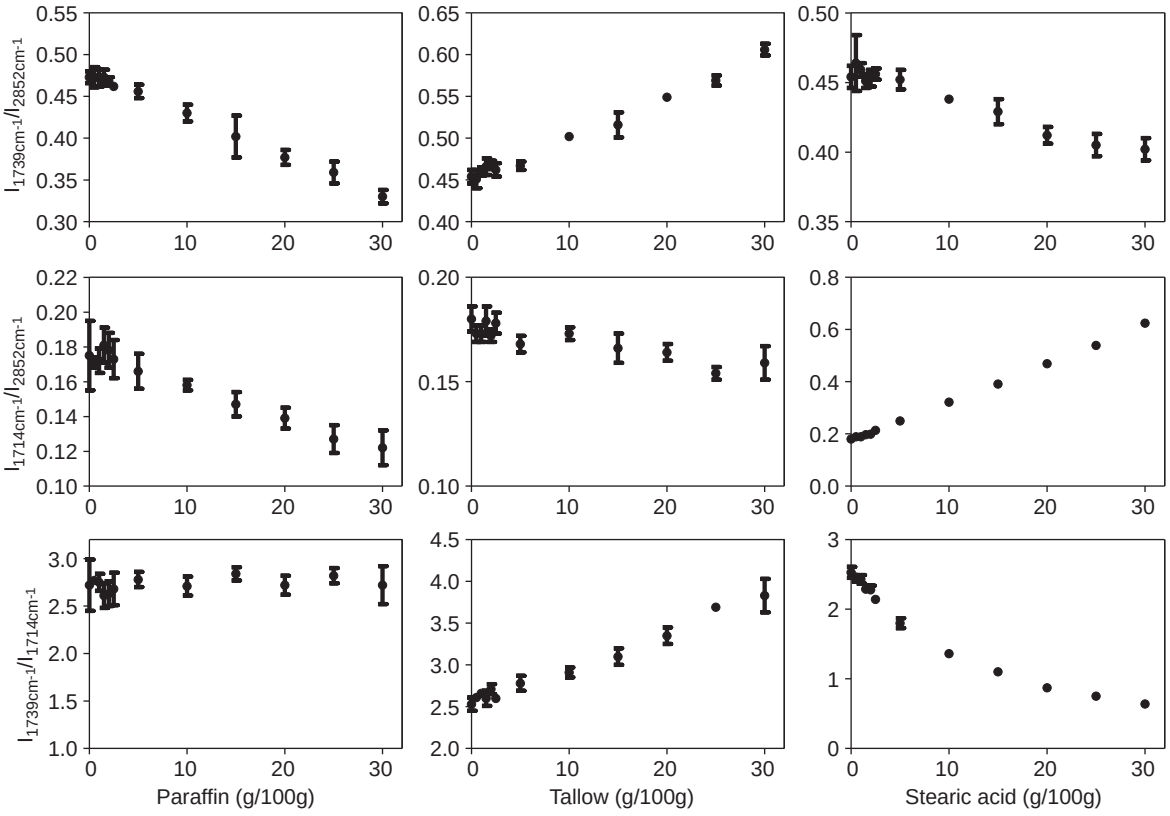

Fig. 2. Change in the $I_{1739\ cm^{-1}}/I_{2852\ cm^{-1}}$, $I_{1714\ cm^{-1}}/I_{2852\ cm^{-1}}$ and $I_{1739\ cm^{-1}}/I_{1714\ cm^{-1}}$ ratios obtained for beeswax intentionally adulterated with paraffin, tallow and stearic acid.

-0.01646 ± 0.00569; $p < 0{,}0057$) in this ratio is observed in relation to that of the original sample. From this concentration up to 30% stearic acid adulteration, a significant linear decrease in this ratio ($I_{1739\ cm^{-1}}/I_{2852\ cm^{-1}} = -0.003490\ C_{Stearic\ Acid} + 0.4736$, $r = 0.550$, $F = 12.2$, $p < 0.0016$) is observed. For the $I_{1714\ cm^{-1}}/I_{2852\ cm^{-1}}$ ratio, post-hoc analysis shows that the first concentration of added stearic acid where a significant difference is observed is 1.5% (mean difference 0.01669 ± 0.00476; $p < 0{,}0010$).A significant linear increase in the $I_{1714\ cm^{-1}}/I_{2852\ cm^{-1}}$ ratio is observed with increasing amounts of stearic acid added between 1.5% and 30% ($I_{1714\ cm^{-1}}/I_{2852\ cm^{-1}} = 0.01534\ C_{Stearic\ Acid} + 0.1605$, $r = 0.995$; $F = 3501$, $p < 0.0001$). For the $I_{1739\ cm^{-1}}/I_{1714\ cm^{-1}}$ ratio, post-hoc analysis shows that the first concentration of stearic acid where a significant difference is observed is 0.5% (mean difference -0.0773 ± 0.0305; $p < 0{,}015$). For this concentration, a significant quadratic decrease in this ratio is observed ($I_{1739\ cm^{-1}}/I_{1714\ cm^{-1}} = 2.526(C_{Stearicacid})^2 - 0.1243 C_{Stearicacid} + 0.00207$, $r = 0.986$). Based on the obtained results, the stearic acid adulteration of beeswax at concentrations greater than 0.5% can be easily determined using the $I_{1739\ cm^{-1}}/I_{1714\ cm^{-1}}$ ratio. Additionally, the ratio $I_{1714\ cm^{-1}}/I_{2852\ cm^{-1}}$ shows a good detection limit (1.5%) for the stearic acid adulteration of beeswax.

These results show that the use of these proposed ratios allows beeswax adulteration by paraffin (and microcrystalline wax) at concentrations greater than 5%, tallow at concentrations greater than 5% or stearic acid at concentrations greater than above 0.5% to be easily detected. These detection limits are lower than that attainable by the classical analytical methods (10% for paraffin, 2% for stearic acid and 10% for tallow adulteration) [6] and are similar to the detection limits observed for the previously developed chromatographic methods (1%–4% for each adulterant) [5,12,14]; this comparison demonstrates the power of the ATR–FTIR method for the detection of beeswax adulteration.

3.3. Establishment of critical values of beeswax authenticity and performance of the method

For the ATR–FTIR method to have real applications for the detection of beeswax adulteration, the upper and lower limits of each ratio for authentic beeswax samples need to be established. Traditionally, virgin beeswax is taken as a beeswax purity standard. Nevertheless, during the commercial processing of beeswax, which is necessary for its recycling and purification [31,32], high temperatures are employed, which can result in changes in the beeswax composition [3]. Additionally, as beeswax ages and darkens, its *n*-alkane composition changes [33]. The amount of even-numbered *n*-alkanes (C22–C32) increases in darker-colored beeswax compared to that in light-colored beeswax, most likely because of the accumulation of cuticular residues from bees found in the darker-colored comb cells. For these reasons, the analysis of virgin beeswax samples as an authenticity standard may result in an overestimation of the number of intentionally adulterated beeswax samples. Nevertheless, virgin beeswax is the only authentic beeswax standard available, and it was therefore used as a starting point to establish the authenticity limits for the $I_{1739\ cm^{-1}}/I_{2852\ cm^{-1}}$, $I_{1714\ cm^{-1}}/I_{2852\ cm^{-1}}$, and $I_{1739\ cm^{-1}}/I_{1714\ cm^{-1}}$ ratios. Thirty-one independent virgin beeswax samples were analyzed using the proposed methodology, and the values obtained for the $I_{1739\ cm^{-1}}/I_{2852\ cm^{-1}}$, $I_{1714\ cm^{-1}}/I_{2852\ cm^{-1}}$ and $I_{1739\ cm^{-1}}/I_{1714\ cm^{-1}}$ ratios are presented in Table 1. As evident from the results, the values obtained for the virgin beeswax were very similar: a relative standard deviation of only 3.33% was observed for the $I_{1739\ cm^{-1}}/I_{2852\ cm^{-1}}$ ratio and deviations of 5.51% and 5.47% were

observed for the $I_{1714\ cm^{-1}}/I_{2852\ cm^{-1}}$ and $I_{1739\ cm^{-1}}/I_{1714\ cm^{-1}}$ ratios, respectively.

To establish the critical values, the first significant differences observed in the intentionally adulterated beeswax were added and subtracted from the maximum and minimum values obtained during the analysis of virgin beeswax, respectively (Section 3.2). The proposed upper and lower critical values are presented in Table 1. This approach to establishing the upper and lower critical values can limit the detection power of the method for adulterated beeswax because of the natural variation in the higher and lower values for the virgin beeswax for the absorbance ratios, which were 0.071, 0.047 and 0.487 for the $I_{1739\ cm^{-1}}/I_{2852\ cm^{-1}}$, $I_{1714\ cm^{-1}}/I_{2852\ cm^{-1}}$ and $I_{1739\ cm^{-1}}/I_{1714\ cm^{-1}}$ ratios, respectively. For example, a beeswax sample presenting a value of 0.532 for the $I_{1739\ cm^{-1}}/I_{2852\ cm^{-1}}$ ratio before adulteration, if adulterated with 5% paraffin, would present a value of 0.515 and would still be classified as authentic; therefore, the proposed critical ranges can be conservative. To study the applicability of the proposed method to real samples, test the proposed critical limits, and detect the most common point of beeswax adulteration in the commercial beeswax production chain, 45 beeswax sheets and 49 comb beeswax collected in the Portuguese market, which represent the industrial transformation of beeswax and the collection of beeswax by the beekeepers, respectively, were analyzed using the proposed methodology. The results obtained are shown in Fig. 3. The spectra of both the beeswax sheet and comb beeswax samples exhibited ratios outside the proposed critical ratios, especially for the $I_{1739\ cm^{-1}}/I_{2852\ cm^{-1}}$ ratio; these results show that the samples were adulterated with paraffin/microcrystalline wax. Only one comb beeswax sample exhibited a higher $I_{1739\ cm^{-1}}/I_{1714\ cm^{-1}}$ ratio and was suspected of being contaminated with tallow; nevertheless, the other parameters for this sample were inside the proposed ranges. No adulteration with stearic acid was detected in any of the analyzed samples. Additionally, a large number of samples exhibited ratios between the lower critical limit and the lower range observed for the virgin beeswax samples. For beeswax sheets and comb beeswax samples, 60% and 22% of the samples were found to be adulterated with paraffin/microcrystalline wax, respectively. Based on these figures, paraffin is concluded to be the most common beeswax adulterant used in the Portuguese market, and beeswax adulteration is a major concern in beekeeping.

The intermediate precision of the proposed ratios was obtained by collecting the ATR–FTIR spectra of the sheets and comb beeswax samples on five different days. The precisions were not dependent on the level of the measured ratio and were

Table 1
Values of the $I_{1739\ cm^{-1}}/I_{2852\ cm^{-1}}$, $I_{1714\ cm^{-1}}/I_{2852\ cm^{-1}}$ and $I_{1739\ cm^{-1}}/I_{1714\ cm^{-1}}$ absorbance ratios obtained for the virgin beeswax analyzed by ATR–FTIR.

	IR absorbance ratio		
	$I_{1739\ cm^{-1}}/I_{2852\ cm^{-1}}$	$I_{1714\ cm^{-1}}/I_{2852\ cm^{-1}}$	$I_{1739\ cm^{-1}}/I_{1714\ cm^{-1}}$
Average	0.498	0.196	2.556
Standard deviation	0.017	0.011	0.140
Relative standard	3.33%	5.51%	5.47%
Median	0.499	0.196	2.540
Maximum	0.532	0.215	2.807
Minimum	0.461	0.168	2.320
Upper critical limit	0.55	0.23	3.1
Lower critical limit	0.44	0.15	2.1

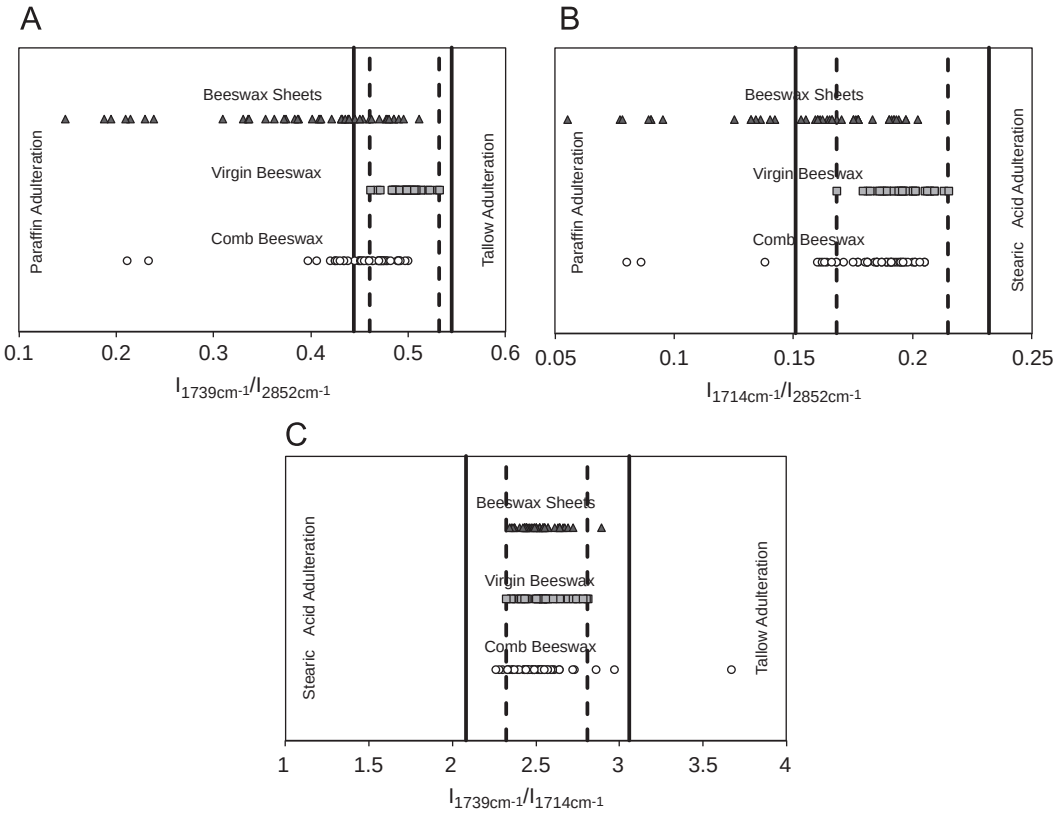

Fig. 3. The $I_{1739\ cm^{-1}}/I_{2852\ cm^{-1}}$, $I_{1714\ cm^{-1}}/I_{2852\ cm^{-1}}$ and $I_{1739\ cm^{-1}}/I_{1714\ cm^{-1}}$ ratios obtained for the analysis of virgin (■), sheet (▲) and comb (O) beeswax samples collected in the Portuguese market. Solid vertical lines represent the upper and lower critical values for the ratios, and dashed vertical lines represent the minimum and maximum values obtained in the analysis of virgin beeswax.

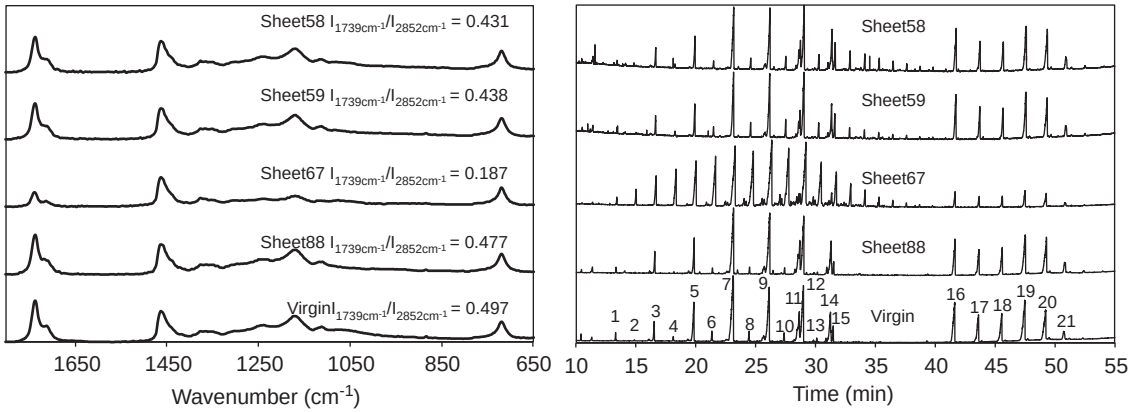

Fig. 4. Representative examples of the results obtained by the ATR–FTIR method on real beeswax samples and analysis of the same samples by HT-GC-FID; hydrocarbons: 1—heneicosane, 2—docosane; 3—tricosane; 4—tetracosane; 5—pentacosane; 6—hexacosane; 7—heptacosane; 8—octacosane; 9—nonocosane; 10—triacontane; 11—hentriacontene; 12—hentriacontane; 13—dotriacontane; 14—tritriacontene; 15—tritriacontene isomer; palmitate monoesters: 16—tetracosyl hexadecanoate; 17—hexacosyl hexadecanoate; 18—octacosyl hexadecanoate; 19—triacontyl hexadecanoate; 20—dotriacontyl hexadecanoate; 21—tetratriacontyl hexadecanoate.

0.0148, 0.00856 and 0.112 for the $I_{1739\ cm^{-1}}/I_{2852\ cm^{-1}}$, $I_{1714\ cm^{-1}}/I_{2852\ cm^{-1}}$ and $I_{1739\ cm^{-1}}/I_{1714\ cm^{-1}}$ ratios, respectively; these results show that the method demonstrates good precision.

To have an independent assessment of the beeswax classification obtained using the ATR–FTIR method, the samples were analyzed by high-temperature GC–FID and classified by unsupervised chemometric analysis [21], and the classification obtained using the ATR–FTIR method was compared. Some illustrative examples are shown in Fig. 4. Sample 88 exhibited a $I_{1739\ cm^{-1}}/I_{2852\ cm^{-1}}$ ratio of 0.477 and was classified as an authentic beeswax. A comparison of the HT-GC/FID chromatogram of this sample with that of the virgin beeswax sample (Fig. 4) reveals that sample 88 is clearly not adulterated with paraffin. Sample 67 exhibited a $I_{1739\ cm^{-1}}/I_{2852\ cm^{-1}}$ ratio of 0.187 and was classified as an adulterated beeswax sheet. The adulteration with paraffin is evident in the HT-GC/FID chromatogram, which shows that a large number of even-numbered hydrocarbons are present in small amounts in authentic beeswax sheets. With one exception, all of the samples classified as adulterated using the HT-GC/FID method [21] were also classified as paraffin-adulterated beeswax samples using the proposed ATR–FTIR method. The exception was sample 58, which exhibited a $I_{1739\ cm^{-1}}/I_{2852\ cm^{-1}}$ ratio of 0.431 and was classified as being adulterated with paraffin using the ATR–FTIR method and as authentic using the HT-GC/FID method. The HT-GC/FID chromatogram of sample 58 is presented in Fig. 4 and is compared with sample 59, which exhibited a $I_{1739\ cm^{-1}}/I_{2852\ cm^{-1}}$ ratio of 0.438 and was classified as an authentic beeswax sample by the ATR–FTIR and HT-GC/FID methods. Careful inspection of the chromatograms (Fig. 4) does not reveal a substantial difference between the two samples. In fact, the area percentage of hydrocarbons and monoesters is similar (total hydrocarbons: even-numbered hydrocarbons: monoesters ratios of 62.27:7.03:37.73 and 60.62:7.22:39.38 for sample 58 and 59, respectively).

The results obtained for the independent assessment of the authenticity of beeswax samples show that the proposed limits are adequate for the classification as authentic and paraffin/microcrystalline-wax-adulterated beeswax samples. Nevertheless, samples near the critical value should be treated carefully regarding their authenticity. Because no beeswax samples adulterated with stearic acid were found and because only one sample contaminated with cow tallow was found, the proposed critical limits for the detection of adulterated beeswax could not be confirmed with real samples.

4. Conclusions

In this work, a novel, direct, reagent-free method for the detection of beeswax adulteration by single-reflection attenuated total reflectance mid-infrared spectroscopy was developed and validated with real samples. The method can detect, with a good detection limit, beeswax adulteration with paraffin/microcrystalline wax (5%), tallow (5%) and stearic acid (0.5%), which are the four most commonly employed beeswax adulterants.

In addition to the method's simplicity with respect to sample handling, the time needed is significantly less (approximately 4 min per sample) than that required by the previously described methods, which are based on chemical analysis and chromatographic techniques. Upper and lower critical limits for beeswax authenticity were established and validated for paraffin/microcrystalline wax adulteration through the independent analysis of real beeswax samples by HT-GC-FID.

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References

[1] A.P. Tulloch, L.L. Hoffman, J. Am. Oil Chem. Soc. 49 (1972) 696–699.
[2] A.P. Tulloch, BeeWorld 61 (1980) 47–62.
[3] A.P. Tulloch, J. Am. Oil. Chem. Soc. 50 (1973) 269–272.
[4] J.W. White, M.L. Riethof, I. Kushnir, J. Assoc. Agric. Chem. 43 (1960) 781–790.
[5] J. Serra, Grasas Aceites. 41 (1990) 69–72.
[6] J.L. Bernal, J.J. Jiménez, M.J. del Nozal, L. Toribio, M.T. Martín, Eur. J. Lipid Sci. Technol. 107 (2005) 158–166.
[7] J.J. Jiménez, J.L. Bernal, S. Aumente, M.J. del Nozal, M.T. Martín, J. Bernal, J. Chromatogr. A. 1024 (2004) 147–154.
[8] J.J. Jiménez, J.L. Bernal, S. Aumente, L. Toribio, J. Bernal, J. Chromatogr. A. 1007 (2003) 101–116.
[9] R. Aichholz, E. Lorbeer, J. Chromatogr. A. 855 (1999) 601–615.
[10] A.P. Tulloch, J. Am. Oil Chem. Soc. 49 (1972) 609–610.
[11] J. Serra, Grasas Aceites. 39 (1988) 333–342.
[12] J.J. Jiménez, J.L. Bernal, M.J. del Nozal, L. Toribio, J. Bernal, Eur. J. Lipid Sci. Technol. 109 (2007) 682–690.
[13] J.J. Jiménez, J.L. Bernal, M.J. del Nozal, M.T. Martín, J. Bernal, J. Chromatogr. A. 1129 (2006) 262–272.
[14] J.J. Jiménez, J.L. Bernal, M.J. del Nozal, M.T. Martín, L. Toribio, Eur. J. Lipid Sci. Technol. 111 (2009) 902–911.
[15] L.E. Rodriguez-Saona, M.E. Allendorf, Annu. Rev. Food Sci. Technol. 2 (2011) 467–483.

[16] F.R. van de Voort, A.A. Ismail, J. Sedman, J. Am. Oil Chem. Soc. 72 (1995) 873–880.
[17] A. Afran, J.E. Newbery, Spectrosc. Int. 3 (1991) 39–42.
[18] M. Safar, D. Bertrand, P. Robert, M.F. Devaux, C. Genot, J. Am. Oil Chem. Soc. 71 (1994) 371–377.
[19] Y.W. Lai, E.K. Kemsley, R.H. Wilson, J. Agric. Food Chem. 42 (1994) 1154–1159.
[20] J. Sedman, F.R. van de Voort, A.A. Ismail, in: M.M. Mossoba (Ed.), Spectral Methods in Food Analysis,, Marcel Dekker, New York, 1999, pp. 397–425.
[21] M. Maia, F.M. Nunes, Food Chem. 136 (2013) 961–968.
[22] A.P. Tulloch, Lipids 5 (1970) 247–258.
[23] M. Kaplan, G. Davidson, M. Poliakoff, J. Chromatogr. A 673 (1994) 231–237.
[24] S.J. Lee, K. Kim, Vib. Spectrosc. 18 (1998) 187–201.
[25] M.D. Guillén, N. Cobo, J. Sci. Food Agric. 75 (1997) 1–11.
[26] M.S.F. Lie Ken Jie, C.H. Lam, S.C. Wong, Chem. Phys. Lipids 28 (1981) 189–196.
[27] S. Wartewig, R. Neubert, W. Rettig, K. Hesse, Chem. Phys. Lipids 91 (1998) 145–152.
[28] P. Tandon, G. Foster, R. Neubert, S. Wartewig, J. Mol. Struct. 524 (2000) 201–215.
[29] F. Kaneko, K. Yamazaki, M. Kobayashi, K. Sato, M. Suzuki, Spectrochim. Acta A 50 (1994) 1589–1603.
[30] G. Meyer, J. SOFW 135 (2009) 43–50.
[31] A.I.R.N.A. Barros, F.M. Nunes, M. Maia, Good Manufacturing Practices of Beeswax (in Portuguese), F N A P—Federação Nacional de Apicultores de Portugal, Lisboa, 2009.
[32] S. Bogdanov, Apiacta 38 (2004) 334–341.
[33] D. Namdara, R. Neumannc, Y. Sladezkid, N. Haddade, S. Weinera, Apidologie 38 (2007) 453–461.