Journal of Apicultural Research

ISSN: 0021-8839 (Print) 2078-6913 (Online) Journal homepage: https://www.tandfonline.com/loi/tjar20

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To cite this article: Alexandra Špaldoňová, Martina Havelcová, Ladislav Lapčák, Vladimír Machovič & Dalibor Titěra (2020): Analysis of beeswax adulteration with paraffin using GC/MS, FTIR-ATR and Raman spectroscopy, Journal of Apicultural Research

To link to this article: https://doi.org/10.1080/00218839.2020.1774152

Journal of Apicultural Research, 2020
https://doi.org/10.1080/00218839.2020.1774152

ORIGINAL RESEARCH ARTICLE

Analysis of beeswax adulteration with paraffin using GC/MS, FTIR-ATR and Raman spectroscopy

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(Received 24 May 2019; accepted 1 February 2020)

It is generally known that beeswax is falsified, and adulterated waxes are increasingly appearing in markets. Gas chromatography/mass spectrometry (GC/MS), Fourier transform infrared and Raman spectroscopy techniques were used to assess changes in the composition of *n*-alkanes, fatty acids, monoesters, methyl esters, hydroxy esters, and carboxylic acids caused by the adulteration of beeswax by paraffin addition. Both the calibration (pure beeswax and paraffin) and commercial samples of wax were used to compare the efficiency of these methods. For this purpose, the ratio of palmitic acid methyl ester to octacosane was used in GC/MS. Using spectroscopic methods, the relative content of carbonyl groups was chosen as the assessment criterion, and quantitative calibration was based on the ratio of the stretching vibration of the $C=O$ bonds and the deformation vibration of the aliphatic C–H bonds. It was found that even a small addition of paraffin to beeswax caused more than a 50% reduction in the ratio, and 10% paraffin resulted in a 17-fold reduction in the ratio with respect to the pure beeswax sample. Given that beeswax processing includes multiple heating and cooling, the influence of temperature and time on the chemical composition of the waxes was also investigated. There was a trend towards a simultaneous decrease in carboxylic acid groups and an increase in hydroxy esters and palmitic acid methyl ester with increasing heating time. However, the changes were small, so the heating process does not affect significantly the quality of beeswax.

Keywords: adulteration; beeswax; FTIR-ATR; GC/MS; heat treatment; n-alkanes; paraffin

Introduction

Beeswax, obtained from bee colonies of *Apis* bees, is part of our cultural heritage. Historically, the production of beeswax has always been an integral part of honey production. Honeycombs were pressed and the wax mouldings were melted. Approximately one half of this wax was returned to beekeeping in the form of wax foundation, and the other half had a variety of food, agricultural, industrial, and artistic applications. Since 1865, honeycomb centrifugation has been used worldwide as the main method of obtaining honey (Johansson & Johansson, 1968) and this has led to the repeated use of honeycombs and separation of wax production from honey production. Some beekeepers prefer to purchase foundation wax rather than undertaking laborious wax production themselves, and many old honeycombs end up not in wax melters but in waste containers or as wax moth food. The consequence of this is a reduced supply of raw beeswax by beekeepers and an increased demand from producers who require beeswax for their products. The price of native beeswax has risen, and adulterated waxes are increasingly appearing on the market. These products are highly sophisticated and commonly use physical and chemical parameters with the same values as natural wax. Natural substances as well as synthetic products have been applied for adulteration. The most common sources are hydrocarbons from paraffins and microcrystalline waxes, triglycerides from palmitic acid, fat and hardened beef tallow, industrially produced fatty acids (palmitic, stearic acid), long chain alcohols (C_{16}-C_{18}), and C_{32}-C_{36} synthetic esters (Bogdanov, 2009, 2016; Waś et al., 2016). Among them, adulteration with paraffin waxes represents the greatest problem due to its wide availability, low price, and physico-chemical properties (Svečnjak et al., 2015). As a result, the quality of beeswax is compromised, making it unusable in some branches of industry, including beekeeping. It is important that the foundations on which the honeycombs will be built are genuine (Svečnjak et al., 2015).

Many studies have been conducted in order to identify authentication parameters for original beeswax, and mostly, methods were based on the physico-chemical parameters of hydrocarbons (Tulloch, 1973). However, the current quality criteria for these procedures (i.e. saponification, density, solubility, melting point, iodine index) are limited and therefore inadequate for a reliable determination, but they still have wide utilization today because of their standardization in different branches, e.g. pharmacy, food industry. Gas

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*Corresponding author. Email: spaldonova@irsm.cas.cz chromatography combined with mass spectrometry (GC/MS) (Jiménez et al., 2003, 2004, 2006; Maia & Nunes, 2013; Waś et al., 2014a, 2014b, 2015, 2016), or high-temperature gas chromatography coupled to a flame ionization detector (HTGC-FID/MS) (Bonvehi & Bermejo, 2012), and spectroscopic techniques such as nuclear magnetic resonance (NMR) (Brüschweiler et al., 1989) and Fourier transform infrared spectroscopy (FTIR) (Edwards et al., 1996; Maia et al., 2013; Svečnjak et al., 2015) have shown better performance in the detection and quantification of various substances in beeswax. In addition to its simplicity with respect to sample handling, the amount of sample, and time needed to perform the analysis, the methods are less ambiguous than those based on physico-chemical analyses (Jiménez et al., 2003, 2004, 2006, 2007; Maia et al., 2013; Pinzón et al., 2013; Svečnjak et al., 2015). However, the authentication of beeswax is not easy because not only adulteration but also ageing or processing can substantially change its composition (Namdar et al., 2007). Beeswaxes are processed in two steps: in a first step the wax is extracted and cleaned, in the second it is purified by melting either by boiling in water, by steam, or by heat from electrical or solar power (Bogdanov, 2004). The heat treatment can result in significant changes in beeswax composition depending on the temperature and heating time (Regert et al., 2001; Tulloch, 1973). This, therefore, complicates analytical interpretations.

All the above chromatographic and spectroscopic methods are commonly used techniques that have already been verified for the detection of beeswax adulteration; nevertheless, these methods have not been compared and contrasted. The objectives of this study were to compare the efficiency of different methods (GC/MS, FTIR-ATR, and Raman spectroscopy) in the detection of beeswax adulteration and to determine the advantages and disadvantages of these analytical procedures for routine detection of beeswax adulteration with paraffin. Due to possible changes in beeswax composition caused by the temperature of industrial recycling of beeswax, the study was complemented by an investigation into the effect of heating on n-alkanes, fatty acids, and esters as a simulation of an integral part of the industrial recycling of beeswax for the production of comb foundation.

Materials and methods

Materials

Calibration samples containing 0–100% (w/w) paraffin were prepared using paraffin (Sigma-Aldrich, Czech Republic) and pure beeswax (reference sample of authentic virgin beeswax): beeswax and paraffin weights were melted and homogenized. The pure beeswax sample was also chosen for cyclic heat treatment. Pure authentic virgin beeswaxes were used for methods validation. A set of study materials covered 15 commercial samples of block wax and wax foundations of an unknown chemical background obtained from different beekeeping shops and stores across the Czech Republic. These samples were collected in the period from 2013 to 2015. All samples were provided by the Bee Research Institute at Dol, Czech Republic.

Elemental analysis and gas chromatography/mass spectrometry

The elemental composition of dried samples was performed on the Thermo Finnigan FLASH EA 1112 Series CHNS/O microanalyzer.

20 mg of a sample was dissolved in 5 ml of dichloromethane (DCM) using a sonication bath until the content was fully dissolved. GC/MS analysis of the total extracts was carried out using a Trace GC Ultra gas chromatography coupled to a DSQ II mass spectrometer (Thermo Scientific). Chromatographic separation was performed on a capillary column DB5 (60 m × 0.25 mm inner diameters, 0.25 μm film thickness). The oven temperature program was as follows: initial temperature of 100 °C, held for 1 min, 5 °C/min ramp to 320 °C, and held for 15 min. The temperature of the injection port was 280 °C and a 1 μl sample was injected in splitless mode. The temperature of the ion source was 230 °C and mass spectra were obtained by scanning from 40 to 650 *m/z* at standard electron energy of 70 eV. For data processing and peak integration, Xcalibur software was applied. Identification of the studied compounds (*n*-alkanes, fatty acids, esters, hydroxy esters, and carboxylic acids) was based on a comparison of spectra from the National Institute of Standards and Technology mass spectral library. The MS detector was operated in the Total Ion Current (TIC) acquisition mode for fatty acids and was combined with the Selected Ion Monitoring (SIM) acquisition mode for *n*-alkanes and methyl esters fatty acids.

For analysis of beeswax total acids 20 mg of wax dissolved in 5 ml of DCM was mixed with 2 ml of methanol and derivatized using 2 ml of reagent 14% BF_3 in methanol. Hydrolysis and methylation were carried out at 90 °C for 1 h. After that, 2 ml of water were added to the mixture, and the vials were shaken manually for 30 s. The aqueous phase was removed and the organic layer was then washed with water twice to remove excess reagents (Jiménez et al., 2003).

All reagents were of pesticide grade. DCM was purchased from the P-Lab company (Czech Republic), and the derivatization reagent and internal standard, 2,6-dimethylundecane, were obtained from Sigma-Aldrich (Czech Republic).

Fourier transform infrared-attenuated total reflectance (FTIR-ATR) and Raman spectroscopy

FTIR-ATR spectra of the bulk samples were collected on a Nicolet 6700 FTIR spectrometer (Thermo Nicolet

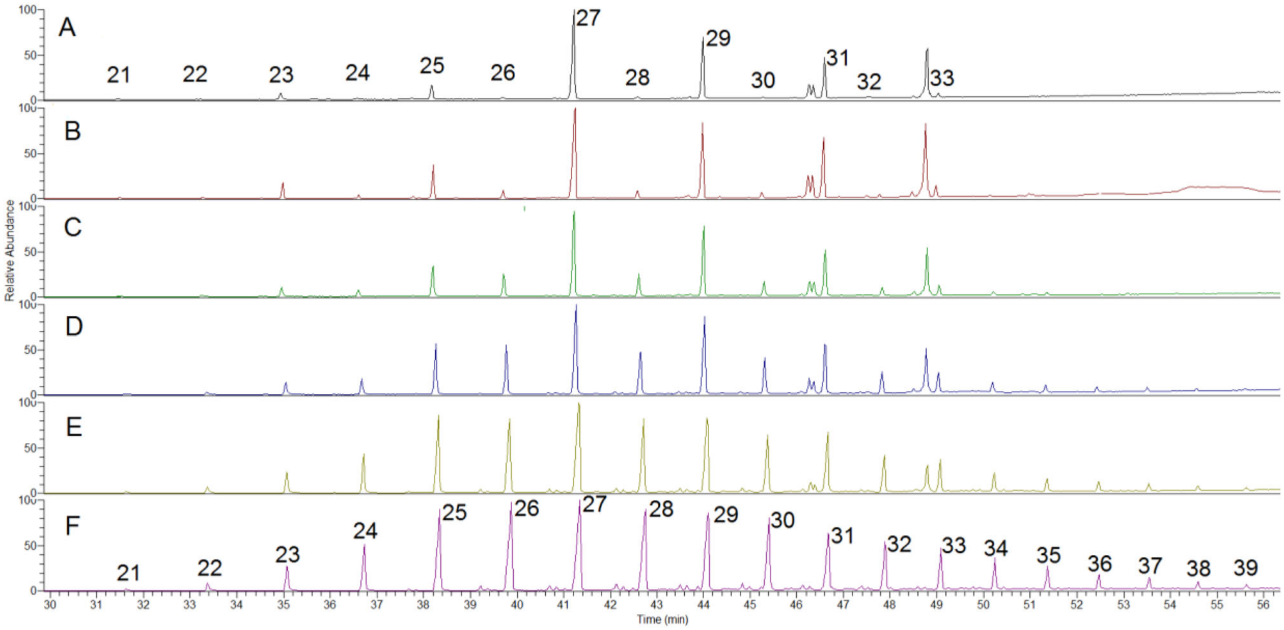

Figure 1. SIM chromatograms showing the distribution of *n*-alkanes (m/z 85): pure beeswax (A), adulterated beeswax with increasing paraffin content (B, C, D, E respectively), and paraffin (F). The numbers above peaks indicate the carbon numbers of *n*-alkanes.

Instruments Co.) with a N_2 purging system. Spectra were acquired using ATR GladiATR accessory equipped with a single bounce diamond crystal (angle of incidence 45°). Each sample spectrum averaged 64 scans and the resolution was 4 cm^{-1}. The spectra were plotted against a single-beam spectrum of the clean ATR crystal and converted into absorbance units by ATR correction. Data were collected over a wave number range 4000–400 cm^{-1}.

Raman spectra were collected using a Thermo Scientific DXR Raman microscope equipped with a 532 nm line laser (power 10 mW). The spot size of the laser focused by the 50× objective was ~1 μm in diameter. The scattered light was analyzed by a spectrograph with holographic grating (900 lines per mm) and a pinhole width of 50 μm. The acquisition time was 10 s. Ten accumulations were added together to obtain a spectrum.

Heat treatment

Pure beeswax was heated in a laboratory dryer to 100 °C with a temperate increase rate of 1.5 °C per minute, held at 100 °C for 60 minutes and then decreased at a rate of 1.5 °C per minute. Ten, thirty, sixty, and one hundred heating cycles were used to obtain samples Alter1, Alter2, Alter3, Alter4, respectively. The Alter5 sample was the result of continuous heating at 100 °C for 144 hours. Prior to GC/MS analysis of the prepared samples, an internal standard (2,6-dimethylundecane) was added in a concentration of 0.5 mg/ml. For a description of quantitative changes in the heated samples, FTIR-ATR areas of bands at 1736, 1724, 1711 and 1698 cm^{-1} were used. Changes in the volumes of esters, hydroxy esters, and carboxylic acids have been described as percentage changes of the band areas compared to the untreated beeswax band areas.

Quality assurance of analytical method

All samples were stored in the dark at 4 °C until they were analyzed. GC/MS quantification of compounds was carried out according to their retention times, mass spectral data, and peak areas of the internal standard. The FTIR-ATR determination of paraffin content in the beeswax was based on the ratio of the stretching vibration of C = O bonds and the deformation vibration of the aliphatic C–H bonds. The integral intensities were used for analysis. To evaluate the precision of the analysis, three replicates of samples were analysed. The relative standard deviations (SD) of the replicates were calculated and the values were below 10 rel.%. In assessing repeatability (precision), the relative SD for pure authentic virgin beeswaxes was also expressed. The F-test was applied to compare two variances using the SD for the replicate analysis of samples by the used methods.

Results

GC/MS analysis

The same homologous series of *n*-alkanes were identified in the sample of pure beeswax and paraffin (Figure 1). Pure beeswax was composed of odd numbered hydrocarbons in the range *n*-C_{21}-C_{33}; the main hydrocarbons were heptacosane (*n*-C_{27}), followed by nonacosane (*n*-C_{29}) and hentriacontane (*n*-C_{31}). Hydrocarbons with an even number of carbon atoms (*n*-C_{22}-C_{32}) were minor

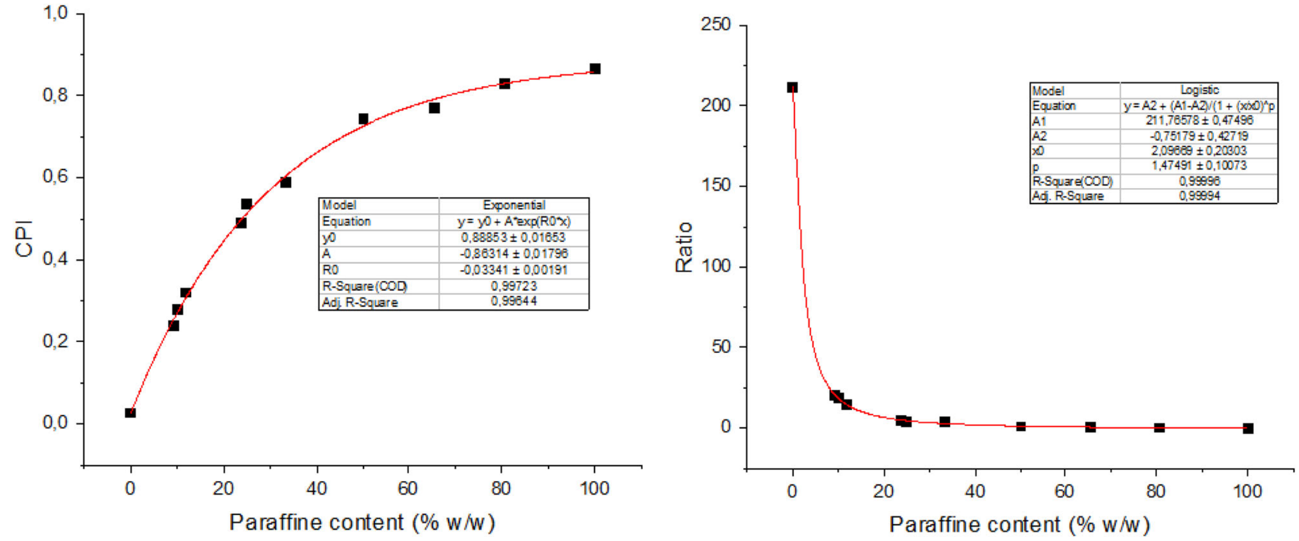

Figure 2. Expression of paraffin content (A) in extracts using CPI, and (B) in derivatized samples using the ratio of palmitic acid methyl ester ($C_{17}H_{34}O_2$) to octacosane ($C_{28}H_{58}$).

components. The addition of paraffin (c_{par}) to beeswax resulted in an increased intensity of peaks with even-numbered hydrocarbons and the occurrence of *n*-alkanes containing more than 33 carbon atoms in the molecule. The samples were characterized using the Carbon Preference Index (CPI = Σ even homologues/Σ odd homologues) counted for the whole range of *n*-alkanes (*n*-C_{21}-C_{33}). The correlation of this ratio with the paraffin content (Figure 2) resulted in an exponential equation that could be shown as linear using the logarithmic scale of paraffin content (Figure 3).

The derivatization procedure, specified in the method section, resulted in the formation of methyl esters of fatty acids in pure beeswax samples whereas, in the paraffin sample, no change occurred due to the missing fatty acids. The chromatographic profile showed a very recognizable pattern of methyl esters of fatty acids with 15 to 37 carbon atoms, of which, palmitic acid methyl ester ($C_{17}H_{34}O_2$) was the major compound at a high concentration (Figure 4). *N*-alkanes with an even number of carbon atoms either were not detected or were at a very low concentration. However, the increasing presence of even-numbered *n*-alkanes with longer carbon chains (*n*-C_{34}, *n*-C_{36}, and *n*-C_{38}) appeared in beeswaxes adulterated by the gradual addition of paraffin.

Relative amounts (%) of palmitic acid methyl ester and octacosane were used in $C_{17}H_{34}O_2/C_{28}H_{58}$ ratios for a correlation with paraffin content (Figure 2). Fitting of this relation led to a logistic function which, when transferred to a logarithmic scale, appeared logarithmic (Figure 3).

FTIR-ATR and Raman spectroscopy analysis

The FTIR-ATR spectra of beeswax were dominated by bands belonging to the antisymmetric and symmetric stretching vibrations of the aliphatic C–H bonds of methyl and methylene groups in the wave number region 3000–2700 cm^{-1} (Table 1). Four bands were distinguished in the spectrum at about 2956 (CH_3), 2916 (CH_2), 2871 (CH_3), and 2848 cm^{-1} (CH_2). The deformation vibrations of aliphatic C–H bonds were represented by intense doublets of scissoring and rocking vibrations at 1472/1462 cm^{-1} and 730/720 cm^{-1} (Figure 5). The splitting of the CH_2 scissoring and rocking modes is indicative of an orthorhombic crystal structure of hydrocarbon chains. The band at 1414 cm^{-1} belongs to the deformation vibration of the methylene group adjacent to the carboxyl group. A low intensity band at 1378 cm^{-1} was due to the symmetrical deformation vibrations of methyl groups.

The FTIR-ATR spectrum of the paraffin wax showed bands in similar positions as the pure beeswax spectrum but did not contain stretching vibration bands of C = O and C–O, and “band progression” features (Figure 5). This difference was used for the determination of paraffin content using the A_{CO}/A_{CH} ratio of the stretching vibration of C = O bonds in the spectral region 1797–1665 cm^{-1} and the deformation vibration of the aliphatic C–H bonds in the spectral region 1496–1423 cm^{-1}. The correlation of this ratio with the paraffin content (Figure 6), resulted in the linear equation $A_{CO}/A_{CH} = 1.578 - 0.0158 \times c_{par}$ ($R^2 = 0.992$).

Raman spectra of pure beeswax and paraffin are very similar. They show prominent bands of the antisymmetric and symmetric stretching vibrations of methylene groups at 2881 and 2847 cm^{-1} (Figure 7). Twisting vibrations of the CH_2 groups (1462, 1440, 1419 cm^{-1}), twisting and rocking vibrations of the same groups (1296 and 1172 cm^{-1}), and symmetric and antisymmetric stretching vibrations of the C–C bonds (1131 and 1063 cm^{-1}) can be also seen in the spectra. A low intensity band at 1655 cm^{-1} was ascribed to the

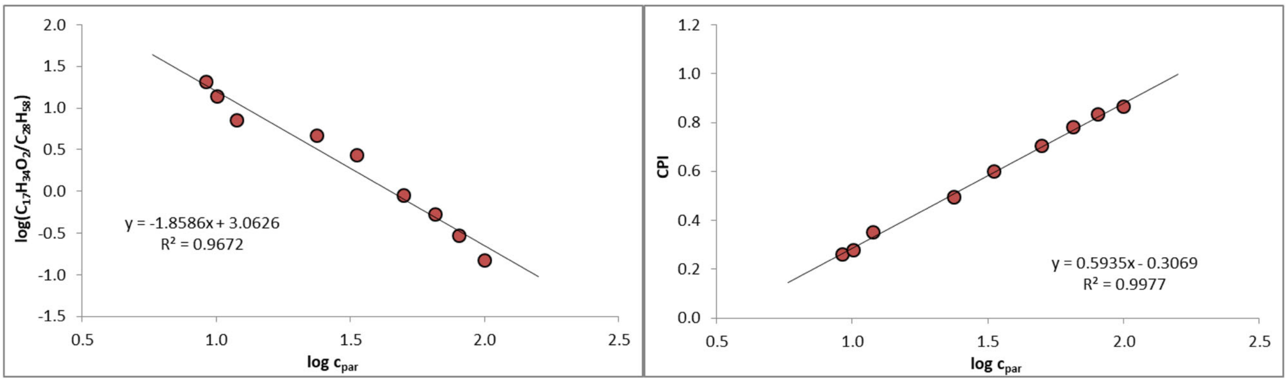

Figure 3. Linear dependence using: a) CPI and a logarithmic scale for paraffin content, b) logarithmic scale for $C_{17}H_{34}O_2/C_{28}H_{58}$ ratio and paraffin content.

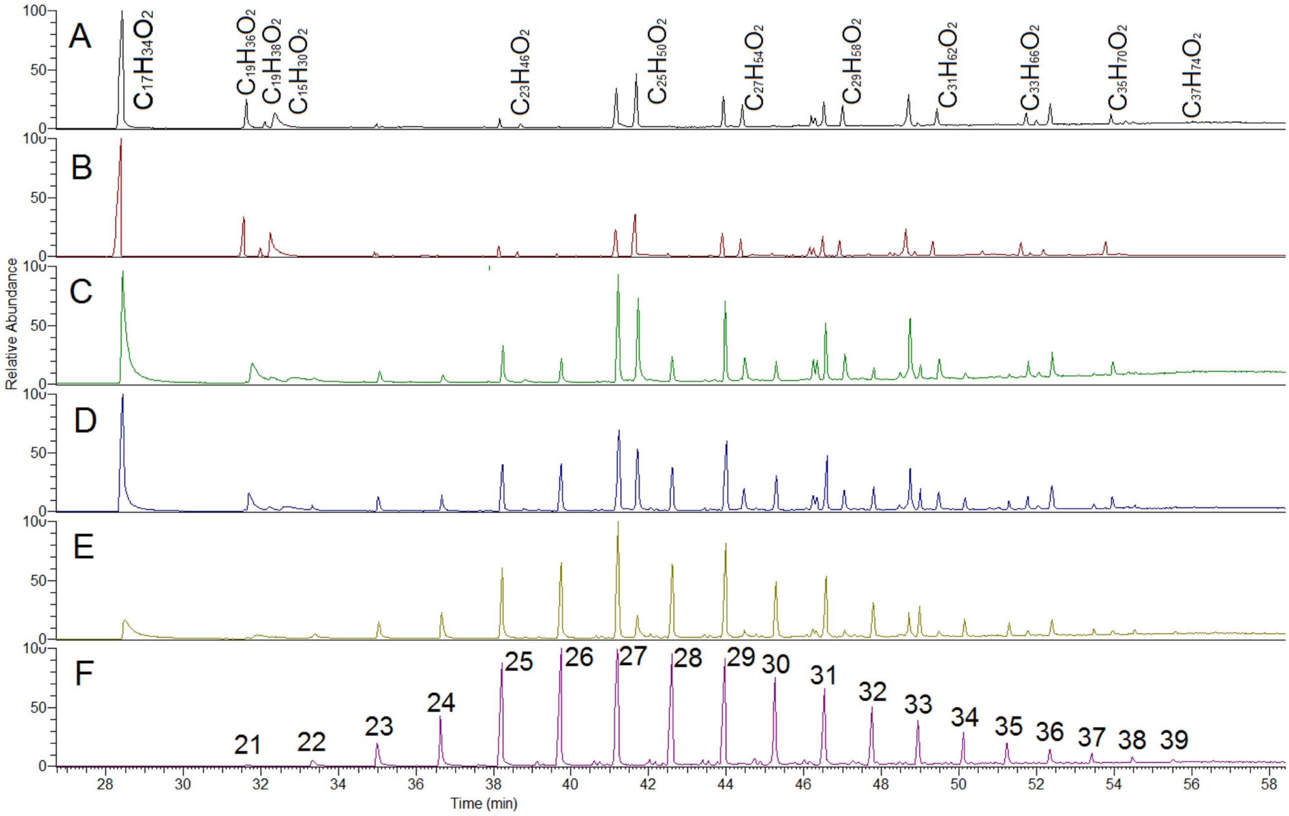

Figure 4. Total ion chromatograms of the samples: pure beeswax (A), adulterated beeswax with increasing paraffin content (B, C, D, E respectively), and paraffin (F) after the extraction of free fatty acids and the corresponding methylation. The numbers above peaks indicate the carbon numbers of *n*-alkanes; the corresponding methyl ester fatty acids are also indicated.

stretching vibrations of C = C double bonds. The band at 1735 cm^{-1} was due to carbonyl groups.

Elemental analysis and methods validation

The beeswaxes adulteration with paraffin was also evident from differences in C, H and O concentrations in beeswax, paraffin, and prepared mixed samples: increasing paraffin content in beeswax resulted in a decreasing concentration of O and a rise in H and C content. This progression was seen in the van Krevelen diagram using the $(H/C)_{at}$ and $(O/C)_{at}$ ratios (Figure 8).

For the validation of all methods, pure beeswax samples and a sample with paraffin at a concentration of 25% (w/w) were used. Using all the obtained equations for each method, the concentrations were calculated and compared with real concentrations (Table 2).

Analysis of commercial beeswax samples

Fifteen commercial samples of real block wax and wax foundations of unknown chemical backgrounds were processed in the same way as the calibration and validation samples. None or minimal paraffin contents - under 5% (w/w) - were found in 11 samples of this set.

Table 1. Assignment of vibrational modes for the FTIR-ATR and Raman spectra of the beeswax and paraffin (Czamara et al., 2015; Edwards et al., 1996; Jones et al., 1952; Kitagawa et al., 1962; Socrates, 2004).

Band position in FTIR-ATR spectrum (cm^{-1})		Band position in Raman spectrum (cm^{-1})		
beeswax	paraffin	beeswax	paraffin	Assignment
2956	2958			$\nu^{as}(CH_3)$
2916	2916	2881	2881	$\nu^{as}(CH_2)$
2871	2872	2932	2932	$\nu^{s}(CH_3)$
2848	2848	2846	2847	$\nu^{s}(CH_2)$
1736		1735		$\nu(C=O)$ esters
1724				$\nu(C=O)$ hydroxyl-esters
1711, 1697	1473, 1462, 1452			$\nu(C=O)$ COOH
		1655		$\nu(C=C)$ olefinic
1472, 1462		1462, 1440	1463, 1441	$\delta(CH_2, CH_3)$
1414, 1397		1419	1419	$\delta(CH_2)$ adjacent to the COOH
1357, 1376	1377, 1368	1371	1370	$\delta(CH_3)$
1345, 1329, 1309, 1288, 1266, 1244, 1220, 1196		1296	1296	$\delta(CH_2)$ wagging + twisting, ν(C–O) at COOH
1171				ν(C–O) esters
		1172	1172	$\rho(CH_2)$
1125	1126, 1106, 1045, 1009			ν(C–O) alcohols
1098, 1114		1131, 1099	1134, 1111	ν(C–O) alcohols, esters ν(C–C)
1051		1063	1063	ν(C–O) alcohols ν(C–C)
1029, 1014				ν(C–O) alcohols
956	959, 889			γ(OH) acids
916		920		
		891	891	$\rho(CH_3)$
730, 720	730, 719			$\gamma(CH_2)$
683				δ(COO) acids
550				ω(COO) acids

ν - stretching, δ – deformation, γ - out-of-plane, ρ – rocking, ω – wagging, as – asymmetric, s –symmetric.

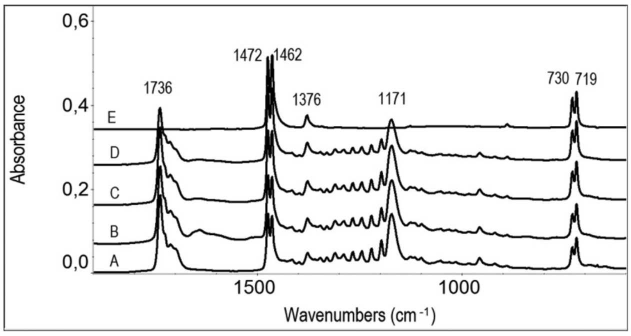

Figure 5. FTIR-ATR spectra of beeswax (A), paraffin (E) and their mixtures 10 (B), 25 (C) and 50 (D) % (w/w) of paraffin.

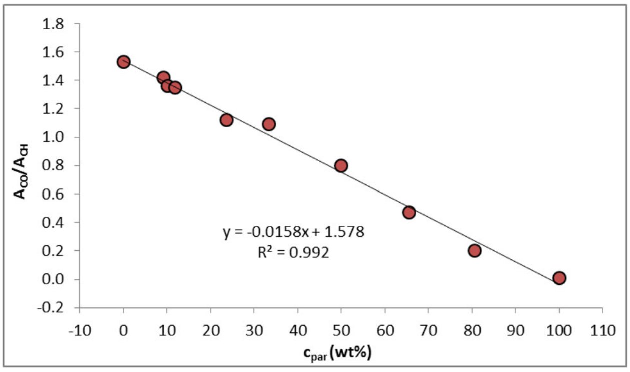

Figure 6. Correlation of the ratio of carbonyl stretching vibrations integrated band area and band area of aliphatic deformation C-H vibrations (A_{CO}/A_{CH}) with the paraffin content (c_{par}).

In the remaining 4 samples of beeswaxes, a substantially high paraffin content - over 50% (w/w) - was determined (Table 3).

Heat treatment

The FTIR-ATR spectra of untreated (Figure 9(A)) and heat-treated pure beeswaxes were very similar. To see differences in details, the bands were separated using mixed Gaussian-Lorentzian functions and their areas were expressed as a percentage of the total separated area between 1800 and 1640 cm^{-1}. The changes in the volumes of esters, hydroxy esters, and carboxylic acids have been described as percentage changes of the band areas compared to the untreated beeswax band areas. Although the changes with the application of heat were relatively small (−2.5–3.0%), there was a visible decrease in the carboxylic groups COOH-2 (band at 1711 cm^{-1}) and an increase in the hydroxy esters OH–COOR (1724 cm^{-1}) (Figure 9(B)). One hundred

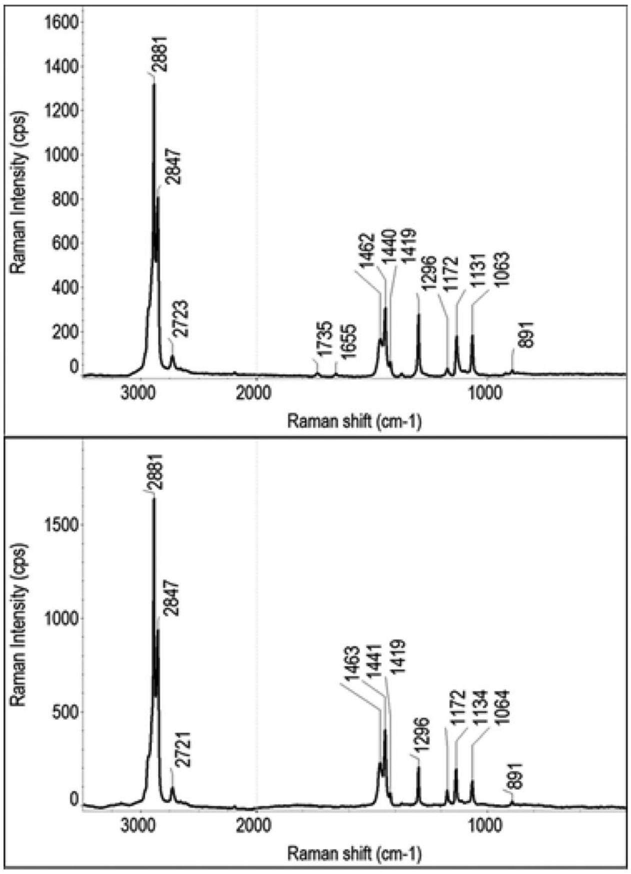

Figure 7. Raman spectra of pure beeswax (upper) and paraffin (lower).

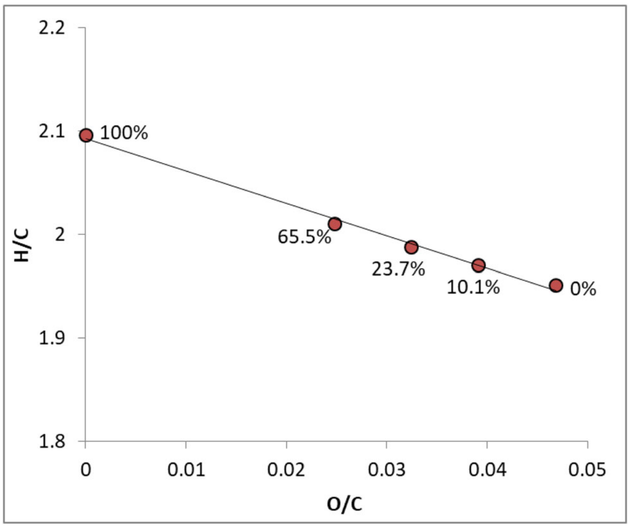

Figure 8. van Krevelen diagram showing atomic H/C and O/C ratios in beeswax samples with different paraffin content. The percentages correspond to the content of paraffin.

heating cycles (Alter4) revealed an increase in esters at the expense of carboxylic acids COOH-1 (band at 1698 cm^{-1}). The sample heated just once to 100 °C for 144 hours (Alter5) demonstrated similar changes.

Using GC/MS, no qualitative or quantitative changes in *n*-alkanes were observed in the heat-treated pure beeswaxes, but an increase in the content of palmitic acid methyl ester ($C_{17}H_{34}O_2$) was evident (Figure 10), which is in agreement with the results of FTIR-ATR analyses.

Discussion

Beeswax is a natural product and no additives are permitted. Determination of sensory and physicochemical characteristics does not guarantee that the wax has not been adulterated, although in some cases they can give hints on possible adulteration. Today adulteration is mostly detected by gas or liquid chromatography to determine the components of the wax (Aichholz & Lorbeer, 2000; Jiménez et al., 2003, 2004). Paraffin is the most used adulteration material, and although there are paraffins available that differ in physico-chemical properties and composition, they all contain an homologous series of *n*-alkanes, having more than 35 carbon atoms in the molecule and an almost identical content of even- and odd numbered alkanes (Waś et al., 2016). This feature is usable in detection of adulterated beeswax because pure beeswax reveals distinctive odd numbered *n*-alkane predominance over even numbered *n*-alkanes, and the peak intensities of the even numbered *n*-alkanes increased with rising representation of paraffin present in the beeswax. This effect has been previously reported (Bonvehi & Bermejo, 2012; Jiménez et al., 2007; Waś et al., 2015) and is confirmed in this work as well (Figure 1). The main features of genuine beeswax are a regular progression of odd carbon-number hydrocarbons and generally there is a maximum in the proportion of heptacontane ($C_{27}H_{56}$) to the other members of the hydrocarbon series. The chain length usually ranges from about C_{21} to C_{33} and those hydrocarbons with even carbon number chains are essentially absent. A simple solution of the beeswax sample in dichloromethane in combination with GC/MS is generally sufficient to detect the presence of paraffin because hydrocarbons fingerprint, typical for paraffin can be observed in the chromatogram.

Moreover, beeswax quality was expressed mathematically using CPI values in an equation. It could be concluded that the CPI values below 0.1 correspond to a strong odd-numbered predominance of *n*-alkanes in pure beeswax. Bonvehi and Bermejo (2012**)** also applied the CPI ratio in their work, and classified the values > 0.10 as adulteration with $\geq 5\%$ of paraffin.

Derivatization of samples allowed the detection of fatty acids in pure beeswax and to see their absence in paraffin. Actually, this principle was applied to show the presence of the most intensive palmitic acid methyl ester ($C_{17}H_{34}O_2$) and octadecane ($C_{28}H_{58}$), and was used to express their ratio. This ratio is 0 for paraffin and approaching the value 210 for pure beeswax (Figure 2(B)). The relationship with paraffin content was also expressed mathematically, in a logistic equation.

Table 2. Validation of FTIR-ATR and GC/MS methods using standard variations calculated from measured and real concentrations of the sample.

Real sample composition, content of paraffine (% w/w)	FTIR-ATR		GC/MS			
			CPI		Ratio	
	Result	*SD (%)*	Result	*SD (%)*	Result	*SD (%)*
0	**−0.3**	*1*	**0.2**	*2*	**0.1**	*1*
0	**−1**	*1.3*	**−0.2**	*1.5*	**2.4**	*2.2*
0	**0.2**	*1.3*	**0.2**	*1.4*	**1.8**	*1.6*
0	**0**	*0.9*	**0.1**	*1.3*	**1.7**	*1.5*
0	**1.3**	*1.5*	**0.2**	*1.5*	**1.6**	*2.9*
25	**25**	*5.3*	**26.9**	*5.8*	**26.4**	*6.1*
Ratio = $C_{17}H_{34}O_2/C_{28}H_{58}$						
F-test results:	**FTIR-ATR × CPI**		$F = 1.08 < F_{crit} = 5.05$			
	FTIR-ATR × Ratio		$F = 1.21 < F_{crit} = 5.05$			

Table 3. List of 15 commercial samples of real block wax and wax foundations analysed by FTIR-ATR and GC/MS, and their detailed characterization using the CPI value and the $C_{17}H_{34}O_2/C_{28}H_{58}$ ratio, and their standard variations.

	FTIR-ATR		GC/MS			
			CPI		Ratio	
	Result	*SD (%)*	Result	*SD (%)*	Result	*SD (%)*
1	**2.8**	*5.1*	**0.2**	*2.4*	**0.3**	*2.5*
2	**3.3**	*3.0*	**0.5**	*2.5*	**3.2**	*2.5*
3	**3.5**	*2.4*	**0.5**	*2.9*	**3.9**	*2.1*
4	**4.7**	*3.2*	**2.0**	*2.6*	**2.6**	*2.6*
5	**1.7**	*2.7*	**−0.2**	*2.9*	**1.9**	*2.8*
6	**2.6**	*3.6*	**−0.1**	*2.4*	**2.5**	*2.0*
7	**3.0**	*2.7*	**−0.1**	*2.9*	**0.2**	*2.9*
8	**2.3**	*2.3*	**−0.1**	*2.9*	**1.2**	*2.8*
9	**3.0**	*2.4*	**−0.1**	*3.1*	**0.1**	*2.4*
10	**2.2**	*2.2*	**0.51**	*3.1*	**2.7**	*4.7*
11	**92.1**	*5.7*	**108.2**	*8.9*	**95.9**	*8.6*
12	**79.4**	*5.6*	**80.6**	*5.8*	**75.7**	*6.2*
13	**1.2**	*2.5*	**0.2**	*2.2*	**2.0**	*2.5*
14	**73.4**	*5.6*	**50.7**	*5.6*	**55.4**	*5.1*
15	**51.8**	*5.7*	**47.1**	*5.1*	**50.7**	*5.2*
Ratio = $C_{17}H_{34}O_2/C_{28}H_{58}$						
F-test results:		**FTIR-ATR × CPI**		$F = 1.68 < F_{crit} = 2.48$		
		FTIR-ATR × Ratio		$F = 1.74 < F_{crit} = 2.48$		

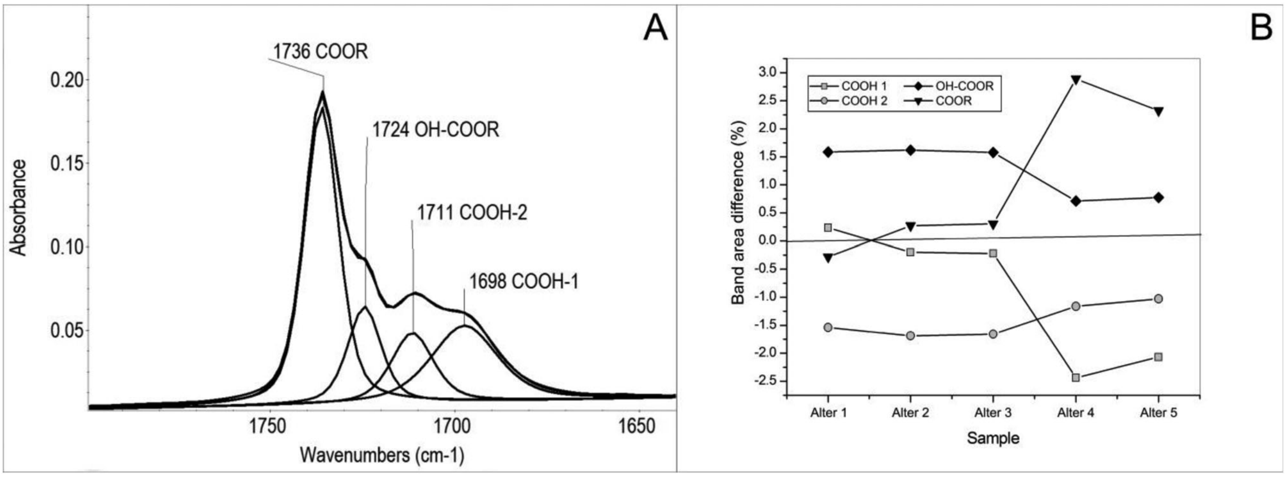

Figure 9. Curve-resolved FTIR-ATR spectrum of pure untreated beeswax (A), and dependence of band area differences (% of band areas of untreated beeswax) upon the different degrees of sample alteration (B). The samples were heated to 100 °C, held for 60 minutes, and then decreased and with ten (Alter1), thirty (Alter2), sixty (Alter3), and one hundred (Alter4) heating cycles; Alter5 sample was heated just once to 100 °C for 144 hours.

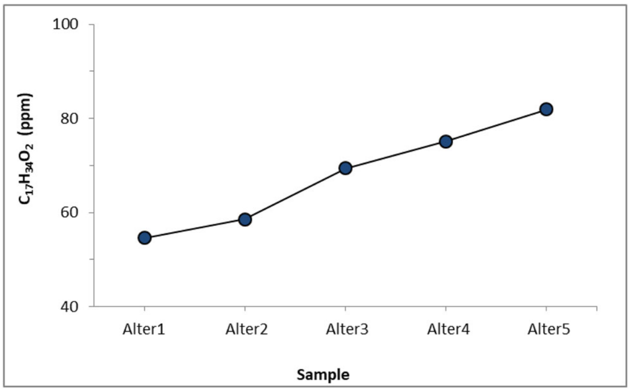

Figure 10. The concentration of palmitic acid methyl ester ($C_{17}H_{34}O_2$) in heated pure beeswax samples (Alter1 – Alter5). Quantification was performed using an internal standard.

Infrared spectroscopy does not allow specific identification of compounds but it permits the determination of functional groups in a sample (Movasaghi et al., 2008). The main absorption bands observed in the infrared spectrum were related to the principal beeswax components – hydrocarbons, monoesters, and free fatty acids (Table 2). Missing bands of the carbonyl groups in the paraffin infrared spectra is a characteristic feature that can be used to detect adulteration. Relative to the bands of aliphatic C–H bonds, this formed the A_{CO}/A_{CH} ratio and was correlated with the paraffin content, resulting in a linear equation.

Both FTIR-ATR and GC/MS methods gave suitable analytical profiles. From the practical point of view, infrared spectroscopy has an advantage due to its simplicity, and time saving; the technique is not destructive and doesńt require extraction or sample pretreatment. The disadvantage of this method is the impossibility to detect exact chemical compounds in the case of other admixtures being present together with paraffin. Correlation coefficients above 0.96 were obtained for all mathematically fitted curves. Validation of the analytical methods was carried out by analysis of 6 samples tested in triplicate. The accuracy of the methods, determined through calculation of the percent deviation, was below 10% but better values were obtained for FTIR-ATR. The minimum detectable percentage using the methods described here was estimated to be 3%, a value that is in agreement with other authors (Waś et al., 2016).

These methods were applied to 15 commercial samples. Although there were differences in values determined for paraffin content using infrared spectroscopy and GC/MS, all methods assessed that a paraffin content $\geq$ 40% (w/w) was present in 4 samples within this set.

The F values were calculated using two-sided F-test at the 95% confidence interval, and all the observed values were less than critical F values. It means that the test assumed that all errors were independent of concentration and used methods.

The other spectroscopic method tested, Raman spectroscopy, couldńt conclusively distinguish between beeswax and paraffin samples due to the low intensity of carbonyl bands and overall similarity of spectral features between samples. Conversely, the elemental composition could distinguish samples adulterated with paraffin but the differences in elemental contents within samples were small and too inaccurate to determine the purity of the beeswax. Moreover, real wax samples can have various elemental compositions.

An investigation of the effect of heating on *n*-alkanes, acids, and esters was carried out as a simulation of heat treatment applied during the industrial recycling of empty combs from the hives. This processing, using a high temperature and heating time, is necessary for purifying beeswax, and possible changes in beeswax composition have already been documented (Tulloch, 1973). Similarly, Tulloch, 1980 in his work mentioned that the low acid values could be caused by the decline of free acids and esters as a result of the reaction of free acids and esters with the secondary hydrocarbons under prolonged overheating. High ester values and high ratio numbers of ester to acid values are also associated with prolonged overheating of the beeswax **(**Tulloch, 1980**).** Five samples, prepared by applying various temperatures and heating times to pure beeswax, did not show any differences in the distribution or concentration of *n*-alkanes. However, a gradual increase in palmitic acid methyl ester was observed (Figure 10). Regert et al. (2001**)** reported that additionally, very low amounts of even-numbered long-chain linear alcohols could appear. The separated bands at 1736, 1724, 1711, and 1698 cm^{-1} used to see changes in the volumes of esters, hydroxy esters, and carboxylic acids showed small but progressive changes in heat-treated pure beeswaxes. Notably 60 or more cycles showed a stepwise increase in esters content at the expense of COOH-1 carboxylic acids (band at 1698 cm^{-1}). The modification of chemical profiles in beeswax, characterized by acquiring palmitic acid methyl ester and hydroxy ester groups, may be attributed to partial hydrolysis of the wax esters. A similar trend was presented by Tulloch, 1973 in his beeswax oxidation study, with the conclusion that free acids presumably react with hydroxy mono- and polyesters, when beeswax is heated above 150 °C. In our case, the oxidation changes at 100 °C were very small even for a very long period of heating, so it can be assumed that oxidation doesn't cause a considerable error in FTIR-ATR analysis of beeswaxes as the bands' ratio, A_{CO}/A_{CH}, used in this method varied for the heat-treated samples over a range of only −0.91–1.91%.

Conclusions

Using GC/MS, added paraffin as an adulterant can be simply ascertained from chromatograms as an increase in chromatographic peak heights of even numbered *n*-alkanes. The ratio of palmitic acid methyl ester and octadecane and CPI values were even more useful in discriminating between genuine and paraffin-adulterated beeswaxes.

Using FTIR-ATR, the ratio of the stretching vibrations of carbonyl bonds and deformation vibrations of the aliphatic bonds were chosen as a parameter for the quantitative determination of the paraffin content in beeswax. Raman spectroscopy was not appropriate to prove adulteration of beeswax due to a low intensity of carbonyl bands and overall similarity of spectral features of beeswax and paraffin therefore, from a practical point of view, there is no reason to further use or develop Raman spectroscopy method.

Almost half of the commercial samples of real block wax and wax foundations obtained from different beekeepers across the Czech Republic had chromatographic and spectroscopic profiles that differed from that of a reference sample of authentic beeswax, indicating their mixing with paraffin. These results point to an urgent need for routine quality calibration of beeswax in the Czech Republic. Statistical comparison of the results obtained by the methods and using the F-test indicated no significant difference with respect to accuracy and precision.

Using GC/MS, no qualitative or quantitative changes in *n*-alkanes were observed in chromatograms after the heat treatment of the beeswax sample. However, a gradual increase in the concentration of palmitic acid methyl ester was found with an increasing number of heating cycles. FTIR-ATR detected changes in abundance of carboxylic acids and esters: the carboxylic acid groups decreased and the hydroxy esters increased with increasing heating cycles.

Acknowledgements

The authors are grateful to John Brooker for reviewing and correcting the manuscript.

Disclosure statement

No potential conflict of interest was reported by the authors.

Funding

The work was carried out thanks to the Operational Program Prague—Competitiveness, the project Centre for Texture Analysis (CZ.2.16/3.1.00/21538), and to the long-term conceptual development of the research organisation RVO: 67985891. The study was supported by the Technology Agency of the Czech Republic, Project No. TA04020318.

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