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SOME ASPECTS OF BEESWAX HYDROLYSIS

SUMMARY

Beeswax is the secretion of a bee's abdominal glands. According to its chemical composition, beeswax belongs to the group of lipids and it is formed a very complex composition of about 300 organic substances. It mostly contains different classes of esters, of which the majority are monoesters. Alkaline hydrolysis of esters (saponification) is a significant chemical reaction in the chemical industry. The saponification value together with the melting point, the acid value and the ester value represent the basic physico-chemical parameters for determining the quality, authenticity and potential faking of beeswax. Saponification has not been fully researched, nor is it an efficient process. Many researchers examine the reaction of ester hydrolysis in order to obtain a process with as little energy consumption as possible, a higher yield and a shorter duration of the hydrolysis process. The main factors that affect the wax saponification process are the size and structure of the alcohol and acid that make up the ester, the wax/alkali mass (molar) ratio, the duration of the process and the temperature of the reaction. With an increase in the mass of the wax sample, i.e. an increase in the wax/alkali mass ratio, the saponification value, i.e. the degree of hydrolysis, decreases. With an increase in the duration of hydrolysis and temperature, the degree of hydrolysis of wax esters, increases. The highest saponification value (SV=79.3; degree of hydrolysis 76.5%) was achieved by the hydrolysis of 0.5 g of wax at a temperature of 105°C for 150 minutes.

Keywords: beeswax, saponification, esters, hydrolysis, saponification value, acid value

INTRODUCTION

Beeswax, produced by honeybees as an excretion from the abdominal glands, is a complex mixture of organic compounds. According to its chemical composition and properties, it belongs to the group of lipids.

The uses and significance of beeswax are very diverse. It is used in various fields of medicine and pharmacy, and in the production of cosmetics, but also in art, during religious ceremonies, as an indicator of environmental pollution, for the production of foodstuffs, in construction, in various branches of the

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engineering industry, and in various other fields. (Ebrahim, 2015, Svečnjak *et al.*, 2019). One characteristic property of wax is that it can preserve its properties unchanged for thousands of years.

Waxes are, for the most part, monoesters of higher fatty acids with higher monohydroxyl alcohols. Fatty acids and higher alcohols usually have an even number of C-atoms and feature a straight chain. In addition to esters, waxes also contain a significant proportion of impurities (often more than 50%) consisting of free fatty acids, oxy-carboxylic acids, free alcohols, hydrocarbons, resinous substances and various others (Ebrahim, 2015, Svečnjak *et al.*, 2019). The properties of natural waxes are not determined by the properties of the esters that make them up, but rather by the properties of the impurities, most notably in terms of their share.

According to its chemical structure, beeswax is a complex mixture that includes over three hundred different substances. The most abundant constituent is myricyl-palmitate, $C_{15}H_{31}CO\text{-}O\text{-}C_{30}H_{61}$. However, there is considerable variation in the chemical composition of beeswax. This is to be expected considering that the chemical composition of the wax depends on the prevailing geographical and climatic conditions, the species and genetics of the bees, the age of the wax and other similar factors. Based on the analyzes of Tulloch (1980), Hepburn (1986), Aichholz and Lorbeer (1999), Negri *et al.* (2000), Garnier *et al.* (2002), Kuzene (2013), and Ebrahim (2015), beeswax contains, in general terms, 35-45% esters, 5.6-14% diesters, 3% triesters, 4-9.5 hydroxymonoesters, about 8% hydroxy-polyesters, 1% acid esters, 2% acid polyesters, about 12% free polyesters, 12-23.5% hydrocarbons, 3.6-18% free fatty acids, 0.6-4.3% free higher alcohols, and about 6% other substances.

In recent times, more than 15 different natural and industrial waxes have been created on the basis of the adulteration of beeswax around the world (Bogdanov, 2004, Svečnjak *et al.*, 2015). Among them, paraffin waxes represent the most significant problem, due to their wide availability and low cost. In addition, paraffin is a white or colorless substance, and is also odorless and chemically inert, which makes it very suitable for adulteration. Occasionally, other unwanted substances, such as stearic acid, stearin, tallow, microcrystalline wax, and others, are also noticed in beeswax.

Determining the physico-chemical properties of beeswax is necessary to determine both its quality and authenticity. The recommendation of the International Honey Commission (IHC) is that the basic physicochemical parameters for quality assessment and the detection of possible adulteration are the melting point, the acid value, the saponification value, the ester value and the ester/acid ratio.

Determining the physicochemical properties of beeswax, in order to determine its authenticity, has both advantages and disadvantages. The advantages include the fact that it can be done using inexpensive equipment, accessories and reagents. It is also easy and quick in terms of its application and no special training is required for the chemical analysis. Furthermore, there is an obvious and clear set of reference values for pure beeswax, which makes comparison straightforward. The disadvantages include the insufficient reliability of certain characteristics when determining the authenticity of beeswax, and the high limit of detection of unwanted impurities (5-50%), Moreover, the analysis process is often destructive and requires a larger amount of sample, meaning that it is necessary to carry out a larger number of analyses in order to determine the presence and the extent of any adulteration to the beeswax.

MATERIAL AND METHODS

The determination of the melting point. The melting point was determined automatically using an instrument based on a capillary tube (a Melting-Point Meter KSPII). The instrument contains a microprocessor to allow for a controlled increase in temperature. Melted beeswax was introduced (approximately 1 cm in total) into a capillary with a length of 10 cm and an inner diameter of 2 mm. The test tube was kept at room temperature for about 24 hours with the solidified beeswax. Then the sample was slowly heated at a rate of 1°C per minute. The melting temperature was the one at which the beeswax was completely melted, which means that it was completely transparent with no cloudiness.

The determination of solubility. In order to test the solubility of the beeswax, at room temperature, 1 ± 0.0001 g of the tested beeswax was introduced into 7 glasses of 250 cm^3. 50 ± 0.05 cm^3 of each solvent was added in each of the glasses in the following order: petrol ether, a mixture of higher alkanes (C_9-C_{15}), mixture of xylene, cyclohexane, acetone, chloroform and ethyl ethanoate. The glasses were hermetically sealed and left at room temperature for 24 hours with occasional mixing.

The determination of the acid value. The acid value (AV) of the wax is the number of milligrams of KOH required to neutralize the free fatty acids in 1 g of beeswax.

We measured 3 ± 0.0001 g of wax in a 250 cm^3 erlenmeyer flask and added 50 cm^3 of solvent, composed of equal parts of ethanol and xylene. We heated the solution in a water bath (at about 60°C) until it dissolved, and then cooled the solution to room temperature. After that, we added 2-3 drops of phenolphthalein and titrated with a 0.1 M ± 0.0001 alcoholic solution of KOH. The analysis procedure was repeated three times.

The acid value was calculated according to the equation:

$$AV = \frac{c_{KOH} \times V_{KOH} \times 56.1}{w_{beeswax}}$$

where: C_{KOH} is the concentration of the KOH solution in $moldm^{-3}$, V_{KOH} is the volume of the used KOH solution in cm^3 and *w* is the mass of the measured wax in grammes.

The determination of the saponification value. The saponification value (SV) is the number of milligrams of KOH required to neutralize the total fatty acids in 1g of wax. The saponification value was determined according to the method prescribed by the European Pharmacopoeia 7th Edition of 2008. A wax mass of 2.0 g ± 0.0001 g was measured in a 250 cm^3 erlenmeyer flask. 30 cm^3 of an ethanol: xylene mixture (1:1 V/V) was added to the wax sample. After that, it was heated in a water bath until it dissolved, and 25.0 cm^3 of standard alcoholic solution KOH, c = 0.5 ± 0.0001 M, was added from a burette. Hydrolysis was performed with reflux for 3 hours. The hot solution was titrated with 0.5 ± 0.0001 M HCl solution, with phenolphthalein as an indicator. The control sample was prepared and titrated in the same way.

The saponification value was calculated according to the equation:

$$SV = \frac{c_{HCl} \times (B - S) \times 56.1}{w_{beeswax}}$$

where c is the molarity of the HCl solution, in $moldm^{-3}$, B is the number of cm^3 of HCl solution used for the control sample, S is the number of cm^3 of HCl solution used for the sample, and w is the weight of the sample in grammes. In our experiments, in order to test the degree of hydrolysis, we measured three different masses of wax: 0.5 g, 1.0 g and 2.0 g ± 0.0001 g. The hydrolysis was carried out with reflux at three different temperatures (85°C, 95°C and 105°C) and over three time intervals: 30 minutes, 90 minutes and 150 minutes.

The ester value (EV) and the ester/acid ratio. The ester value is defined as the amount of KOH in milligrams needed to neutralize 1 g of ester linked acids of beeswax, being calculated by subtracting the saponification value from the acid value: EV = SV – AV. This method is intended to give a measure of the esterified fatty acids in beeswax.

The ester/acid ratio is the quotient of the ester and acid values of individual measurements. According to the International Honey Commission (IHC, 2016), the ester/acid ratio should be in the range of 3.3-4.3.

RESULTS AND DISCUSSION

The melting point of beeswax. The determination of the melting point is not a reliable analytical method for determining beeswax adulteration. For example, paraffin wax is often added to beeswax for adulteration, because it has a similar melting point to beeswax. The melting point of beeswax is not constant, because the composition of the wax varies depending on its origin. Moreover, waxes melt without decomposition and harden again without changing. In our work, the melting point of authentic wax was determined from 5 measurements and is in the range of 62.3-65.4°C. The mean value of the measurement and the standard deviation was shown to be 63.8±1.79°C. The measured value of the melting point is in line with the reports from other literature on the expected value for authentic beeswax (Bernal *et al.*, 2005, Serra Bonvehı and Ornantes Bermejo, 2012, Maia and Nunes, 2013). The melting point of beeswax is not constant, because its composition varies depending on its origin. Various pharmacopoeias offer an expected range of 61–66°C or, more commonly, 62–65°C (Serra Bonvehı and Ornantes Bermejo, 2012). However, even values within this range are not a guarantee of wax purity. Small amounts of impurities change the melting point. If more than 20% of the total mass of the beeswax is in the form of stearic acid, carnauba wax or microcrystalline wax, the melting temperature increases, reaching on average above 65°C. Conversely, when the wax is contaminated with paraffins to a degree greater than 10% of the total mass, the melting point decreases to below 61°C (Svečnjak *et al*., 2015).

Solubility in organic solvents. Beeswax is insoluble in water and resistant to many acids, but it is soluble in most organic solvents, including aromatic hydrocarbons, chloroform, ethers, esters and ketones (Breed *et al.*, 1995, Akoh and Min, 2008, Hossain *et al.*, 2009, Endlein and Peleikis, 2011). According to the European Pharmacopoeia (EP, Council of Europe, 2007) beeswax is practically insoluble in water, partially soluble in hot alcohol (90% V/V) and completely soluble in fatty and essential oils. The solubility of waxes is strongly temperature dependent. At room temperature, beeswax is not completely soluble in any of the above-mentioned solvents. However, when heated, after reaching the melting temperature, it is completely and easily dissolved (Endlein and Peleikis, 2011). Hossain *et al.* (2009) investigated the structure of beeswax using scanning electron microscopy, and found that beeswax contains many crystalline and semi-crystalline compounds. In our tests, the solubility of beeswax at room temperature in the tested organic solvents decreases in the following order: chloroform (97.2%) > a mixture of xylene (93.0%) > cyclohexane (90.3%) > petrol ether (75.0%) > a mixture of higher alkanes, C_9-C_{15} (66.7%) > ethyl ethanoate (36.3%) > acetone (13.7%).

The acid value. The minimum and maximum value of the acid number of the examined beeswax are 15.1 and 18.2, respectively. The mean value ± standard deviation is KN=16.7 ± 1.55. The presence of other substances of an acidic nature can lead to an error in the determination of this value. Slightly higher values of the acid number of authentic beeswax, compared to ours, are shown in the works of certain authors (Bernal *et al.*, 2005, Aguilar *et al.*, 2007, Maia and Nunes, 2013, Svečnjak *et al.*, 2015). Based on a comparative review of the quality parameters of beeswax according to FAO (2005), European legislation (2009/10/EC; EP, 2007) and the International Honey Commission (Bogdanov, 2016), the generally accepted range of acid number values is 17-24(22). Assuming that of the free fatty acids in the wax, only palmitic acid is present, then the content of fatty acids in the examined beeswax is 7.63% w/w. If converted to lignoceric (tetracosanoic) acid, the content of free fatty acids would be 11.0% w/w.

Apis mellifera beeswax contains the highest content of free fatty acids (18%) compared to the beeswax of other studied species (Aichholz and Lorbeer, 1999). Of the free fatty acids, saturated monocarboxylic, unbranched acids with a higher number of C-atoms (C-14 to C-36) are the most abundant. Many authors (Tulloch, 1980, Aichholz and Lorbeer, 1999, 2000, Jimenez *et al.*, 2003) state that the most abundant fatty acid in wax is tetracosanoic (lignoceric) acid, while Serra Bonvehi and Ornantes Bermejo (2012) suggest that it is palmitic acid. Of the unsaturated fatty acids, oleic acid (18:1:9) and linoleic acid (18:2:9,12) are the most abundant.

Fatty acids have a role in bee colony recognition for honeybees in the hive (Breed *et al.*, 1995, Hepburn *et al.*, 2014). Although it is significantly present in the wax, stearic acid does not play a role in the recognition of individuals in the bee hive. Fatty acids play a significant role in the mechanical properties of beeswax, above all by giving it the qualities of resistance and stiffness (Buchwald *et al.*, 2009).

Determining the acid number is a reliable method for determining the contamination of wax by both paraffins and stearic acid. In the case of the falsification of beeswax with the addition of paraffin, the acid value decreases, while the acid value increases with an increase in the content of stearic acid.

The saponification value. The saponification value of the tested sample of beeswax was determined from 3 measurements and was SV = 98.5 ± 6.63. The minimum and maximum values were 92.3 and 105.5, respectively. A similar saponification value for authentic beeswax has also been found by other researchers (Bernal *et al.*, 2005, Maia and Nunes, 2013, Svečnjak *et al.*, 2015). Based on a comparative review of beeswax quality parameters according to FAO (2005), European legislation (2009/10/EC; EP, 2007) and the International Honey Commission (Bogdanov, 2016), the range of saponification values should be 87-102(104). In waxes and lipids in general, the saponification value can be a measure of the average molecular weight of the esters present in the sample. The lower the saponification value, the higher the average molecular weight of the wax esters.

The disadvantage of applying the saponification value in beeswax quality assessment is the variations of certain values reported in the literature (Tulloch, 1973, Serra Bonvehı, 1990, Bernal *et al.*, 2005, Maia and Nunes, 2013, Svečnjak *et al.*, 2015). The deviation of the value of the saponification number is due to exposure to high temperatures (121-140°C) of beeswax used for the construction of honeycombs (Tulloch, 1973, Svečnjak *et al.*, 2019). Therefore, differences in saponification number values can be partially explained by heating (>100°C) during beeswax processing (Tulloch, 1973, Svečnjak et al., 2015, Bogdanov, 2016). However, this does not explain the different ranges shown for beeswax samples collected directly from the hives (Bernal *et al.*, 2005, Maia and Nunes, 2013, Svečnjak *et al.*, 2015). Certain variations in the range of saponification values can also be explained by the different geographical origins of the beeswax.

The saponification of beeswax. Saponification is an important chemical reaction in the chemical industry due to the widespread practical application of esters. In the alkaline hydrolysis (saponification) reaction, esters give the corresponding salt of carboxylic acid and the corresponding alcohol. In this reaction, the alkali is both a reactant and a catalyst. The reaction is irreversible, slow and has a low yield. Saponification as a process has not yet been fully researched, nor is it especially efficient. Many researchers have examined the reaction of ester hydrolysis in order to obtain a process with the minimum possible energy consumption, a higher yield and a shorter duration of the hydrolysis process. The main factors that affect the wax saponification process are the size and structure of the alcohol and acid that make up the wax esters, the wax/alkali mass (molar) ratio and the reaction temperature.

Table 1 shows the mean saponification values, under experimental conditions with 3 variables (sample mass, temperature and time).

Table 1. Mean of the saponification value of beeswax from 3 measurements, for the given experimental conditions

time →	30 min	90 min	150 min	temperature
sample mass↓ (wax/alkali mass ratio)				
0.5 g (0.7)	48.1	66.3	72.8	85 ^{0}C
1.0 g (1.4)	44.9	64.6	71.7	85 ^{0}C
2.0 g (2.8)	34.2	58.7	68.7	85 ^{0}C
0.5 g (0.7)	48.7	71.7	77.2	95 ^{0}C
1.0 g (1.4)	45.4	63.3	75.7	95 ^{0}C
2.0 g (2.8)	34.8	61.5	71.5	95 ^{0}C
0.5 g (0.7)	67.0	75.9	79.3	105 ^{0}C
1.0 g (1.4)	58.2	71.9	78.3	105 ^{0}C
2.0 g (2.8)	51.4	69.8	72.2	105 ^{0}C

The obtained experimental saponification values are lower than the actual values. This is to be expected, considering that the hydrolysis was carried out under cooler conditions. With an increase in the mass of the wax sample, i.e. an increase in the wax/alkali mass ratio, the saponification value decreases. Conversely, when there is an increase in the duration of hydrolysis and temperature, the saponification value, i.e. the degree of hydrolysis of wax esters, increases. Increasing the mass of the wax sample from 0.5 g to 1.0 g, (and thus increasing the wax/alkali mass ratio from 0.7 to 1.4) slightly reduces the saponification value. A significantly greater decrease in saponification is observed with a further increase in the mass of the sample, i.e. the mass ratio of wax/alkali. After 30 and 90 minutes of saponification, the influence of increasing the temperature from 95 to 105°C on the degree of hydrolysis is greater. After 150 minutes, the process is reversed, the degree of hydrolysis is higher when the temperature increases from 85 to 95°C. The highest saponification value (SV=79.3) was achieved by the hydrolysis of 0.5 g of wax, at a temperature of 105°C for 150 minutes. The lowest value of the saponification number (34.2) was achieved by hydrolysis of 2.0 g of wax at a temperature of 85°C for 30 minutes.

The ester value. The ester value of the tested beeswax is $EV_{total}=81.8\pm9.32$. In Table 2, the ester values (EV) for different experimental conditions are given. According to European legislation (2009/10/EC; EP, 2007) the range of ester number values should be 70-80, while according to the International Honey Commission (IHC, 2016) the permissible range is 70-90. The result from this work are similar to those found by other researchers (Akoh and Min, 2008, Maia and Nunes, 2013, Svečnjak *et al.*, 2015). Slightly lower results were reported by Aguilar *et al.* (2007) and Serra Bonvehi and Orantes Bermejo (2012). By contrast, Serra Bonvehi (1990), Puleo and Ritt (1992) and Bernal *et al.* (2005) recorded slightly higher results.

In Table 2, the values of the degree of hydrolysis of wax esters in % for different process conditions are given. The degree of hydrolysis (h) was calculated as: $h=EV/EV_{total}$. For the given experimental conditions, the minimum degree of hydrolysis of esters present in the tested sample of beeswax was 21.4%. The maximum degree of hydrolysis was achieved by the hydrolysis of 0.5 g of wax at a temperature of 105°C for 150 minutes. This produced a result of 76.5%. The ester/acid ratio of the studied beeswax is in the range 4.4-4.8. This is slightly higher than the recommendation of the International Honey Commission (IHC, 2016). A slightly larger range of ester/acid ratio values has also been reported by other researchers (Bernal *et al.*, 2005, Svečnjak *et al.*, 2015). The ester/acid ratio values for the set experimental conditions are given in Table 2.

Table 2. Esterification value (EV); degree of hydrolysis (h) and ester/acid ratio (EV/AV)

time →	30 min			90 min			150 min			Temperature
sample mass (wax/alkali mass ratio)	EV	h	EV/AV	EV	h	EV/AV	EV	h	EV/AV	
0.5 g (0.7)	31.4	0.38	1.9	49.6	0.61	3.0	56.1	0.69	3.4	85 ^{0}C
1.0 g (1.4)	28.2	0.34	1.7	47.9	0.59	2.9	55.0	0.67	3.3	85 ^{0}C
2.0 g (2.8)	17.5	0.21	1.0	42.0	0.51	2.5	52.0	0.64	3.1	85 ^{0}C
0.5 g (0.7)	32.0	0.39	1.9	55.0	0.67	3.3	60.5	0.74	3.6	95 ^{0}C
1.0 g (1.4)	28.7	0.35	1.7	46.6	0.57	2.8	59.0	0.72	3.5	95 ^{0}C
2.0 g (2.8)	18.1	0.22	1.1	44.8	0.55	2.7	54.8	0.67	3.3	95 ^{0}C
0.5 g (0.7)	50.3	0.62	3.0	59.2	0.72	3.5	62.6	0.76	3.7	105 ^{0}C
1.0 g (1.4)	41.5	0.51	2.5	55.2	0.68	3.3	61.6	0.75	3.7	105 ^{0}C
2.0 g (2.8)	34.7	0.42	2.1	53.1	0.65	3.2	55.5	0.68	3.3	105 ^{0}C

CONCLUSIONS

The solubility of the examined beeswax at room temperature in the tested organic solvents decreases in the following order: chloroform (97.2%) > mixture of xylene (93.0%) > cyclohexane (90.3%) > petrol ether (75.0%) > a mixture of higher alkanes, C_9-C_{15} (66.7%) > ethyl ethanoate (36.3%) > acetone (13.7%). These results are to be expected.

The minimum and maximum value of the acid number are 15.1 and 18.2, respectively.The mean value ± standard deviation is AV=16.7±1.55. Assuming that of the free fatty acids in the wax, only palmitic acid is present, then the content of fatty acids in the examined beeswax is 7.63% w/w. When converted to lignoceric (tetracosanoic) acid, the content of free fatty acids would be 11.0% w/w.

The saponification value of the tested beeswax sample was: SV = 98.5 ± 6.63. The minimum and maximum values were 92.3 and 105.5, respectively.

The main factors that affect the wax saponification process include the size and structure of the alcohol and acid that make up the ester, the wax/alkali mass (molar) ratio, the duration of the process and the temperature of the reaction. With an increase in the mass of the wax sample, i.e. an increase in the wax/alkali mass ratio, the saponification value, i.e. the degree of hydrolysis, decreases. On the other hand, when there is an increase in the duration of hydrolysis and temperature, the saponification value, i.e. the degree of hydrolysis of wax esters, increases. The highest saponification value (SV=79.3; degree of hydrolysis 76.5%) was achieved by the hydrolysis of 0.5 g of wax at a temperature of 105°C for 150 minutes. The lowest value of the saponification number (SV=34.2; degree of hydrolysis 21.4%) was achieved by the hydrolysis of 2.0 g of wax at a temperature of 85°C for 30 minutes.

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