

ALTERNATIVE METHOD FOR MEASURING BEESWAX CONTENT IN PROPOLIS FROM THE NETHERLANDS

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Abstract

A convenient method has been developed for the separation and determination of beeswax in propolis from beehives located in the Netherlands. The method is based on differences in specific density and involves the adding of water and microwave heating of a propolis sample. After cooling to room temperature, the resulting three phase system provides a full separation of the beeswax from the propolis in the upper layer. This layer can easily be collected to quantify the authentic beeswax content of the raw propolis sample. Confirmation of the identity of beeswax isolated from propolis with this procedure, was obtained using Near Infrared Spectroscopy (NIR).

Propolis samples from 27 Dutch apiaries from various locations were analyzed using this method. The beeswax content varied between 1.0 and 42.5% with an average of 11.1%. This method may also serve for the de-waxing of raw propolis prior to the isolation of biologically active compounds to be used in medicinal products.

Keywords: separation of beeswax from propolis, Near Infrared Spectroscopy, Netherlands, propolis.

INTRODUCTION

Propolis or bee glue is a resinous material that can be harvested from beehives. Honey bees (*Apis mellifera*) collect resin from plant sources; mainly leaf buds and bark exudates of trees. During collection and processing in the beehive, the resin material is masticated as salivary secretions are added. Other products of bee metabolism and pollen residues may be added. For the application of this resinous material in the beehive, this material is generally mixed with beeswax. The final product, called propolis, is a multi-functional product which the bees use for strengthening, cementing, waterproofing and disinfecting the hive (Burdock, 1998). Propolis plays an essential role in bee health, contributing significantly to the bees' social

immunity (Simone et al., 2009; Simone and Spivak, 2010). Propolis has a long history in ancient folk medicine because it also shows activity against human pathogens. The therapeutic properties of propolis have been extensively reviewed (Ghisalberti, 1979; Marcucci, 1995; Banskota et al., 2001; Almeida and Menezes, 2002; Lofty, 2006; Ramos and Miranda, 2007; Sforcin, 2007; Libério et al., 2009; Fokt et al., 2010; Bognadov, 2011; Araujo et al., 2012). Chemically, propolis is a complex substance as it consists of numerous small and large molecular components (Ghisalberti, 1979; Markham et al., 1996; Burdock, 1998; Bankova et al., 2000; Bankova et al., 2002; Bankova, 2009). It is generally stated, that propolis is composed of around 50% resins, 30% waxes, 10% essential oils, 5% pollen, and 5% various

other compounds, e.g. solid debris materials from pollen grain walls and plant fibers (Ghisalberti, 1979; Burdick, 1998). Due to variable parameters such as location of the hive, type of vegetation, seasonal time of collection, and differences between *Apis mellifera* (sub-) species, the composition of propolis is highly variable (Bankova et al., 1998; Salatino et al., 2005).

As indicated by scientific data, the content of wax in propolis is highly variable too, with reported values from 5 to 50% (Farré et al., 2004; Sforcin, 2007; Lozina et al., 2010; Aliboni et al., 2011; Bonvehi and Gutiérrez, 2011). The wax component of propolis is claimed to largely consist of beeswax. The percentage of wax in propolis depends on what the bees use it for. For example, propolis used for the filling up of cracks and holes in the hive has a high wax content (Nakamura and Seeley, 2006).

Beekeepers may collect propolis from places in beehives where it is usually found in high quantities, e.g. at the ears of the frames. For the professional and standardized collection of propolis, special nets or screens are commercially available. These nets have a mesh width of 2 mm and are usually placed on top of the frames in the upper part of the beehive.

For therapeutic use, the biological active ingredients, viz., flavonoids, aromatic esters, and acids are, because of their polarity, extracted from the propolis with a polar organic solvent, usually ethanol or an ethanol/water mixture (Ghisalberti, 1979; Marcucci, 1995; Banskota et al., 2001; Almeida and Menezes, 2002; Lofty, 2006; Ramos and Miranda, 2007; Sforcin, 2007; Libério et al., 2009; Fokt et al., 2010; Bogdanov, 2011; Araujo et al., 2012; Extracts NZ Ltd., 2012). However, the presence of substantial amounts of non-polar beeswax can easily lead to the formation of undesirable emulsions. This is an important limitation as it may hinder the isolation of these compounds from propolis by means of ethanol. Hence, an efficient de-waxing step to facilitate the extraction appears mandatory.

Wax extraction in combination with a confirmation of the identity of the beeswax has been carried out by Negri et al. (1998; 2000). In these studies, the propolis samples were treated with

ethanol in Soxhlet extractors. After cooling, the whitish wax material was isolated and further analyzed by gas chromatography/electro impact mass spectrometry. A large similarity was found in the composition of the extracted propolis-wax and comb-wax, suggesting a common origin for both wax sources.

The authenticity of beeswax can also be determined by using physical-chemical parameters, such as melting point, density, acid value, saponification value, ration number, ester value, iodine absorption number, and peroxide value (Bogdanov, 2004; Bernal et al., 2005). When using sufficient parameters, adulteration percentages down to 5% can be determined.

Both the de-waxing and analytical procedures to confirm the identity of the isolated beeswax are laborious and demand advanced technology (Negri et al., 1998; Negri et al., 2000; Jiménez et al., 2003; Jiménez et al., 2004; Bernal et al., 2005; Jiménez et al., 2007; Bonvehi and Bernejo, 2012).

The aim of our study is two-fold. First we developed a basic and efficient procedure for the determination of beeswax in propolis. Second, we applied our method for an inventory of the quantitative occurrence of beeswax in propolis from apiaries of beekeepers in the Netherlands.

MATERIAL AND METHODS

Earlier pooled samples for preliminary analysis

Prior to the main study (in 2008, 2009, 2010), we collected propolis over some time, at four apiaries. The collected amounts were pooled per apiary. The obtained samples of 100 g ($n = 2$), 180 g, and 200 g were analyzed, respectively, in total, for wax content. The samples were heated "au-bain-marie" (water temperature kept at about 90°C) until the whole sample had become liquid. By slowly cooling the samples to room temperature, the beeswax separated from the dark brown propolis as a top layer of relatively uniform thickness.

Collection of individual samples

Raw propolis samples were obtained from bee colonies of Dutch beekeepers. The samples

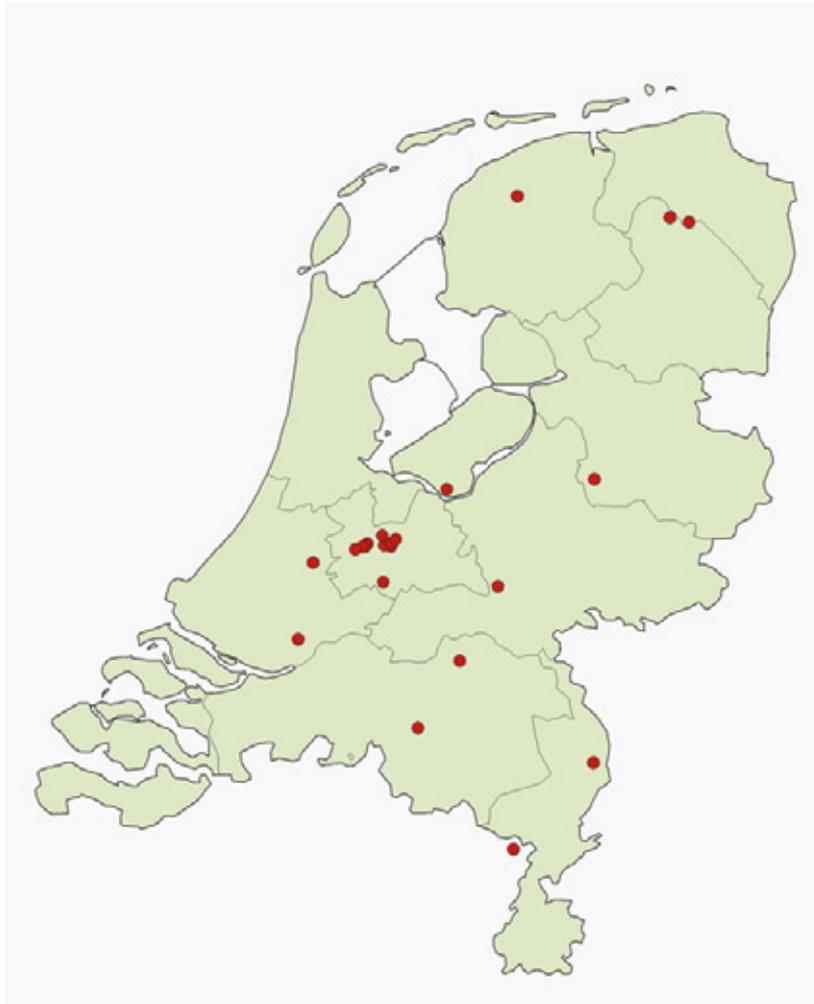


Fig. 1. Apiary locations in the Netherlands from where propolis samples were taken.

were collected by scraping propolis from the ears of the wooden frames and other depositions inside the hive, such as queen excluders and hive-covers.

These 28 samples consisted of two series. The first series was collected from apiaries in the province of Utrecht and the second series was collected from all over the country. Figure 1 shows the locations of the apiaries from which the 28 propolis samples were taken. One sample was collected in Belgium just across the border of Southeast Netherlands. The samples were taken in 2010 and 2011. The weight of these samples ranged between 5 to 50 g. For the validation of the method, a duplicate sample was analyzed for each collected amount above 40 g (15 samples, see Tab. 2).

As reference material for confirmation purposes, we used pure beeswax obtained from comb cappings of the honey harvested in 2009, 2010, and 2011 (apiary Vleuten, 3451 PT).

Preparation of the samples

The individual propolis samples ($n = 28$) were manually chopped at an ambient temperature (ca. 15 - 20°C) in pieces of about 1 - 2 g and mixed; 20.00 g of each sample were transferred into a 50 mL polypropylene tube which had a screw cap. Samples consisting of smaller amounts of propolis, were transferred, in total, to the tube (minimally 5 g).

At a temperature of about minus 4°C, a subset of four samples was firstly ground to powder in a coffee grinder machine. Next, a 50 mL tube which had a screw cap was used to hold 15 g of the powder.

Separation of beeswax and determination of content

The amount of 25 mL of de-ionized water was added to the 20 g of chopped propolis. When adding the water to the powdered sample, it is necessary to constantly and carefully stir

the mixture to avoid propolis powder floating on the water surface. After loosely tightening the screw cap to prevent pressure building up while heating, the tubes were vertically placed in a household microwave apparatus which was set at medium. The time of heating was adjusted so that the temperature rises to about 100°C but without the boiling of the water phase. Depending on the number of samples and their size, the heating time ranged between 1 and 4 minutes. Next, cooling down the samples to room temperature provided a three layer system in the tubes: the beeswax (upper layer), then water (middle layer), and de-waxed propolis at the bottom. With a small stainless steel spatula the beeswax in the upper layer was transferred to a paper tissue for the removal of the remaining water. Next, the amount of extracted beeswax was weighed and the content as a percentage of the weight was calculated.

Instrumental analysis (NIR) of the beeswax in the propolis samples

About 200 mg of the separated wax were put into the bottom of a glass vial. Next, the vial was shortly heated to melting temperature (about 60°C) so that the sample could be put into a vial for the NIR analysis. NIR spectra were recorded on an Antaris II FT-NIR spectrometer (Thermo Scientific, Madison, USA) equipped with an integrating sphere and an InGaAs detector. Measurements were carried out in the diffuse reflection mode with an optical resolution of 8 cm⁻¹ over the spectral range of 10000 - 4000 cm⁻¹, and 32 spectra were co-added. All samples were measured twice. Spectra were compared using Omnic software (version 7.3).

Statistical analysis

A statistical analysis of the duplicate samples calculates the variation of the beeswax content expressed as the relative standard deviation (RSD,) for sample *i*.

$$RSD_i = \frac{\sqrt{\sum (x_{ij} - \bar{x}_i)^2}}{\bar{x}_i}$$

With x_{ij} the *j*th result for sample *i* and

$$\bar{x}_i = (x_{i1} + x_{i2})/2$$

The overall relative standard deviation is calculated by pooling as:

$$\overline{RSD} = \sqrt{\frac{\sum_{i=1}^n RSD_i^2}{n}}$$

(Gort and Hoogerbrugge, 1995).

RESULTS

Preliminary experiments

In the preliminary experiments with pooled apiary samples, the separation was carried out by heating "au-bain-marie" and without adding water to the samples. By separating the top layer above the propolis stratum and subsequently weighing this wax fraction, the beeswax content of these samples could be determined and the results are shown in Table 1.

The beeswax content of these samples ranged between 5 and 12.5% (Tab. 1). Over a two year period, at the apiary of Vleuten (3451PT) the yearly harvest of raw propolis per hive (*n* = 7) was about 60 g.

Table 1.

Beeswax content (% w/w) in pooled propolis season samples			
City and Postal code* of apiary	Season of collection	Amount analyzed (g)	Beeswax content (%)
Utrecht, 3584 AD	2008/2009	100	6.5
Vleuten, 3451 PT	2008/2009	100	8.0
Vleuten, 3451 PT	2009/2010	200	12.5
Zeewolde, 3896LA	2009/2010	180	5.0

*In the Netherlands (NL)

Table 2.

Beeswax content (% w/w) in propolis samples of various locations (n = 28)

Apiary (City, Postal code, Netherlands/Belgium)	Subsample 1	Subsample 2	Average
Duplicate samples			
Deventer, 8121 PR, NL	42.5	49.5	46.0
Dordrecht, 3314 BH, NL	5.0	5.0	5.0
Eelde, 9761 BE, NL	6.5	5.3	5.9
Geffen, 5386 KR, NL	15.5	13.0	14.3
Grubbenvorst, 5971 VX, NL	30.5	27.5	29.0
Kinrooy, 3640, Belgium	27.0	28.5	27.8
Maarsseveen, 3566 JS, NL	4.6	2.6	3.6
Reeuwijk, 2811 GN, NL	2.0	6.0	4.0
Vleuten, 3451 PT, NL	5.5	6.5	6.0
Vleuten, 3451 RD, NL	7.0	6.4	6.7
Zeewolde, 3896 LA, NL	7.8	5.0	6.4
Apeldoorn, 7325 AK, NL*	4.5	4.1	4.3
De Meern, 3454 HE, NL*	1.0	1.0	1.0
Opheusden, 6701 PZ, NL*	21.8	20.6	21.2
Utrecht, 3571 KL, NL*	2.8	3.0	2.9
Single samples			
Groenekan, 3737 RA, NL	2.0		
Harmelen, 3481LH, NL	4.6		
Leeuwarden, 8916 GN, NL	7.5		
Noordlaren, 9479 PB, NL	10.0		
Oirschot, 5688 JX, NL	18.5		
Udenhout, 5071 BJ, NL	5.3		
Utrecht, 3561 PK, NL	5.9		
Utrecht, 3552 CP, NL	11.0		
Utrecht, 3514 XR, NL	12.5		
Utrecht, 3584 AD, NL	6.5		
Vianen, 4132 XE, NL	20.0		
Vleuten, 3451 PP, NL	11.6		
Wageningen, 6707 BN, NL	12.0		
Average of all samples	11.1	12.3	12.3

*Sample ground to powder at a low temperature prior to analysis.

Table 3.

Overview of method performance		
Type of sample preparation	Number of duplicates	Overall relative standard deviation
Manual cutting and mixing at room temperature	11	27%
Grinding at a low temperature	4	4.5%

The individual samples

The samples collected for the inventory of a larger area, contained considerably less material each. This required a modification of the procedure applied for the pooled samples. For reasons of efficiency, the possibility of using a microwave oven for heating was investigated, in combination with a standardized sample size of 20 g.

During our first trials with the simultaneous microwave heating of 4 - 8 samples, an unexpected large difference in temperature increase was observed between individual samples. Even when reducing the heating time to less than 1 min, some samples started boiling. The result was that sample material attached to the underside of the screw cap. After this, microwave heating was combined with the addition of a volume of water to the sample, to smoothen the extraction power. This was successful, and we found that a satisfactory separation was obtained by microwave heating of a sample in a 50 mL tube that consisted of 20 g of homogenized raw propolis to which 25 mL of water was added.

The results are shown in Table 2. The beeswax content of the individual samples collected all over the Netherlands, ranged between 1 and 42.5%. The average beeswax content in the studied population ($n = 28$) was 11.1%. This is within the range of the pooled samples (Tab. 1). As shown in Table 3, the overall relative standard deviation for these 11 duplicate values is 27%. The analysis of the four duplicate samples that were ground to powder at a low temperature resulted in a relative standard deviation of 4.5%.

Confirmation of beeswax

The separation procedure provides a yellowish material that looks very similar to pure beeswax from comb cappings (see Materials).

For the unambiguous confirmation that the material of the separated top layer really consisted of beeswax, NIR spectra were recorded of a randomly selected number of beeswaxes obtained from propolis samples ($n = 7$) as well as of pure beeswax. The NIR spectra of beeswax obtained from the seven propolis samples showed a very high similarity and a large mutual agreement with spectra of the pure beeswax sample. An example is given in Figure 2, showing the NIR spectra of pure beeswax from comb cappings and the beeswax obtained from a propolis sample of the apiary Vleuten (3451PT). The similarity between the spectra unambiguously confirms the similar composition of both waxes.

DISCUSSION

The difference in density between propolis and beeswax can advantageously be used for the separation of these two materials by heating and cooling the raw propolis, with or without the presence of water. The use of water provides a three-layer system consisting of propolis (at the bottom), water (middle layer), and beeswax (floating on top), and, hence, a complete physical separation of the two materials. Microwave heating accelerates the method, rendering a fast, simple and low cost procedure, suitable for small samples.

To confirm that the separated wax from propolis corresponds to pure beeswax, we used NIR technology. This fast and nondestructive technique for comparison or verification of the identity of chemicals is widely used in food and

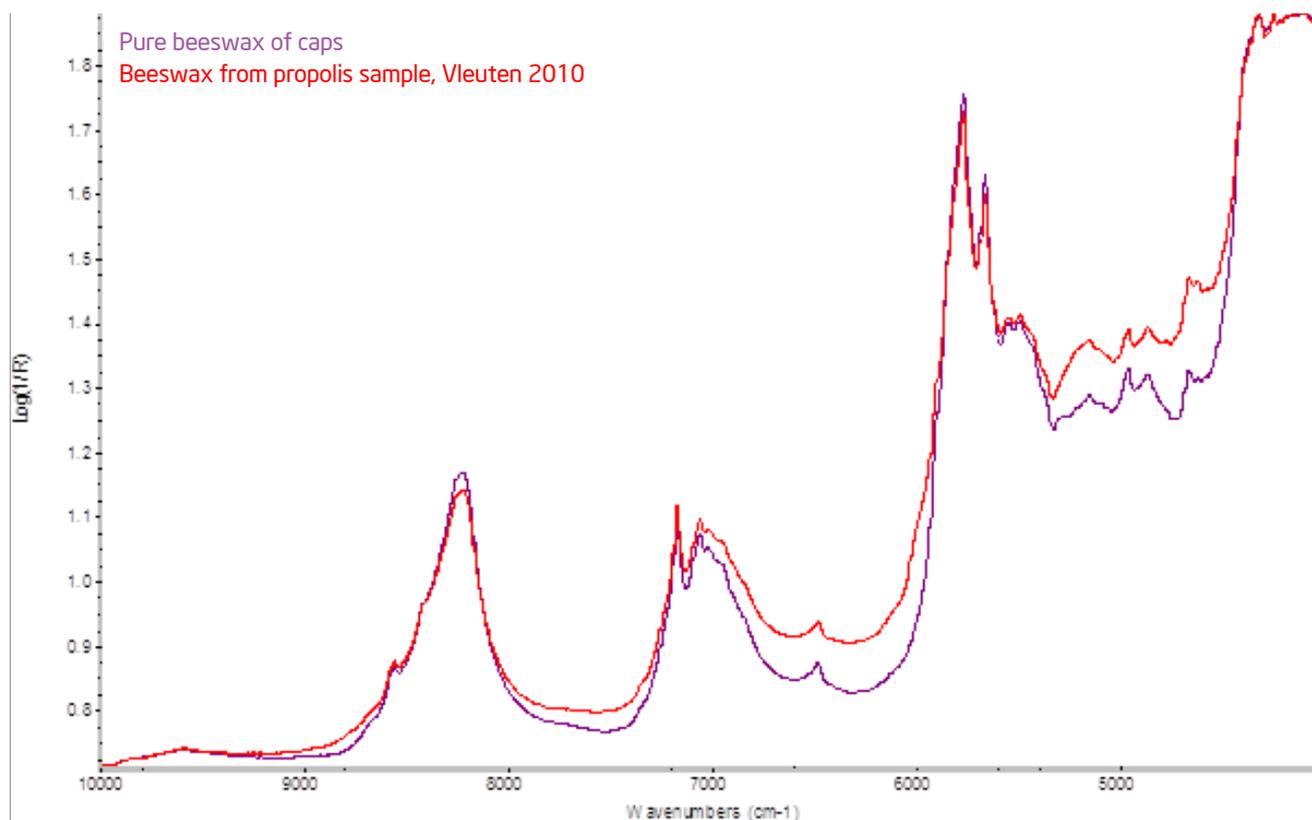


Fig. 2. NIR spectra of beeswax obtained from a propolis sample of apiary Vleuten 3451PT and of pure beeswax obtained from comb caps, both from 2010.

pharmaceutical analysis (Blanco et al., 1998; Qiu et al., 1999; Nicolai et al., 2007). The NIR-results clearly confirmed that the separated solid phase from the raw propolis is beeswax. This proves that our simple separation procedure is in agreement with the sophisticated instrumental analysis of beeswax samples extracted from propolis samples (Negri et al., 1998; Negri et al., 2000; Lozina et al., 2010; Bonvehi and Gutiérrez, 2011).

The variation of the beeswax content in the samples (1 - 42.5%) corresponds with the ranges found in literature of 2 - 30% (Aliboni et al., 2011), 7.5 - 35% (Farré et al., 2004), 2 - 30% (Bonvehi and Gutiérrez, 2011) and 3.8 - 30.6% (Lozina et al., 2010). As mentioned above, the chemical composition of propolis is highly variable due to various reasons - the variation in vegetal origin and location being the most important ones. However, in this inventory the high range in beeswax content is most likely the result of the non-standardized sampling carried out by the individual beekeepers. Propolis collected from different locations in the hive may have different beeswax contents.

The overall performance of the method is given in Table 3. The found standard deviation of 27% for the 11 duplicate samples reflects the difficulty encountered in homogenizing the sample. At room temperature, the raw propolis is very sticky which hampers manual chopping and mixing. Consequently, the variation for the duplicate values is mainly due to sample inhomogeneity. The results obtained for the duplicate samples ground at a low temperature have a relative standard deviation of 4.5%. However, since this analysis is distinctly more laborious and requires specific laboratory facilities (cold room and special equipment), we recommend as a simple analysis methodology, the use of the manual chopping and mixing of collected raw propolis at an ambient temperature.

Our method provides a satisfactory separation of the beeswax from propolis. On one hand, this separation might facilitate the further processing of propolis for therapeutic use. On the other hand, the odorous beeswax separated in this way might render a very attractive product for use in pharmacopoeia and cosmetics (Tihonov et al., 1986).

At the same time, the water phase of the three-layer separation procedure might be of interest in extracting polar biological active compounds rendering an aqueous solution for possible therapeutic application. We cannot exclude that the valuable properties of propolis might change due to the heating process of the method. We experienced that heating the propolis, as applied here for the segregation of beeswax, significantly enhances the lifetime of propolis. Propolis heated for de-waxing and kept under proper conditions did not change in texture and sensory characteristics even after more than 20 years. Without such a preheating step, pulverization of propolis was sometimes observed to start after several months, probably caused by microbial action.

CONCLUSIONS

A simple and efficient method has been developed for the separation of beeswax in raw propolis collected from beehives. With the developed procedure, an inventory of beeswax content in propolis samples from beehives of Dutch apiaries was carried out showing an average beeswax content of 11% with a range between 1.0 and 42%.

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