Raman Spectroscopic Study of Spatial Distribution of Propolis in Comb of *Apis mellifera carnica* (Pollm.)

M. A. STREHLE,[1]* F. JENKE,[1] B. FRÖHLICH,[2] J. TAUTZ,[3] M. RIEDERER,[2] W. KIEFER,[1] J. POPP[1]*

[1] Institut für Physikalische Chemie der Universität Würzburg, Am Hubland, Würzburg D-97074, Germany

[2] Julius-v.-Sachs-Institut für Biowissenschaften der Universität Würzburg, Julius-von-Sachs-Platz 3, Würzburg D-97082, Germany

[3] Theodor Boveri Institut für Biowissenschaften der Universität Würzburg, Am Hubland, Würzburg D-97074, Germany

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ABSTRACT: Micro-Raman spectroscopy and Raman mapping are applied to investigate the spatial distribution and chemical composition of wax and propolis in the comb of *Apis mellifera carnica* (Pollm). A thick layer of propolis at the rim of some cells is identified by Raman spectroscopy. Raman mapping is applied to resolve the distribution of propolis and wax on a micron scale. Both components are connected at the rim of the cell with a mixture of wax and propolis. A layer of almost pure propolis is found on top of the mixture. It appears that even in the mixture, where both components come into close contact, the propolis and the wax remain separated and keep their chemical identity. © 2003 Wiley Periodicals, Inc. Biopolymers (Biospectroscopy) 72: 217–224, 2003

Keywords: Raman mapping; honeybees; propolis; wax; spatial distribution; chemical composition

INTRODUCTION

Propolis is a generic term for natural resinous substances that honeybees [e.g., *Apis mellifera carnica* (Pollm.)] collect from plant and bud exudates.[1] The bees use propolis as a sealer for their hives[1,2] and to mummify creatures that have been killed after invading the hive.[3–5] Propolis exhibits antibacterial, antifungal, and antiviral activity.[5,6] The bees probably disinfect their internal environment by spreading a thin layer on the internal walls of their hive[1] and on the cell walls.[6] Darchen[7] studied the ways of rebuilding the wax comb after an experimental excision of part of it. Chauvin[8] reported that some type of propolis is deposited around the rim of each cell, which inhibits any new construction. Another hypothesis is that the purposeful application of propolis in the comb might play an important role for the structural composition of the comb. This might affect the melting behavior of the waxes and the vibration transmission across the honeycombs, which play an important role in the communication of honeybees.[9]

So far it is not known whether propolis or a propolis–wax mixture is distributed homogeneously or in a certain pattern on the comb. Furthermore, it is of interest to ascertain if propolis and wax form a single phase or two separate phases or if the propolis is chemically modified by

Correspondence to: J. Popp (juergen.popp@uni-jena.de).
**Present address:* Institut für Physikalische Chemie der Universität Jena, Helmholtzweg 4, Jena D-07743, Germany.
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the honeybees. For example, one phase can be a mixture of wax and digested propolis.

In the last decade Raman spectroscopy has been developed as a powerful tool for studying biological samples such as plant and animal tissue,[10–12] wax and resin,[13,14] and honey.[15] In order to obtain spatially resolved information from the sample, Raman mapping or imaging can be used.[16–18]

In this article we report on Raman spectroscopic investigations of the chemical composition and spatial distribution of propolis in honeycombs. Distinct areas of the comb sample have been investigated by taking Raman spectra with a spatial resolution of $\sim$1 μm. Samples of wax and propolis, as well as examples of representative compounds of both components, have been investigated by Raman spectroscopy.

MATERIALS AND METHODS

The Raman spectra were taken with a micro-Raman setup (Labram, Jobin Yvon). The spectrometer has a focal length of 300 mm and is equipped with a 950 lines/mm grating. The spectral resolution was about 4 cm^{-1}. The 633-nm line of a He:Ne laser with a laser power of 20 mW was used as the excitation wavelength. An Olympus MLPlanFL 50 objective focused the laser light onto the sample. A motorized *xy* stage was applied for Raman mapping, a technique that yields information on the spatial distribution of the different substances that are located on the surfaces of the comb sample. The spot size of the laser was $\sim$1 μm and the step size was 0.5 μm.

For a characterization of the chemical composition of the wax and propolis, the most abundant main components, as well as synthetic mixtures of these components in order to simulate the original wax and propolis, were analyzed separately.

Middle-aged wax (2–3 years old) originating from *A. mellifera carnica* (Pollm.) consists mainly of long chained aliphatic compounds with chain lengths ranging from C_{21} to C_{54}. The wax is composed of organic esters (47 wt %), alkanes (15 wt %), alkenes (8.8 wt %), alcohols (0.74 wt %), and acids (0.51 wt %).[19] As representative examples for these substance classes we chose stearylstearat (Fluka) for wax esters, pentacosane (Fluka) for wax alkanes, 1-eisocene (Sigma) for alkenes, triacontanol (Fluka) for alcohols, and tetracosanic acid (Fluka) for acids.

The bulk spectra of the standard substances were taken from the dry powdered substances. To minimize crystalline effects the measurements were taken on different points on the sample. All spectra were of a similar quality.

Propolis from *A. mellifera carnica* (samples from Hannover, Germany) consists mainly of flavonoids, esters, aromatic and aliphatic acids, and other aromatic compounds.

As representative examples to mimic the composition of propolis we used galangin (21.6% total ion current [TIC]) as a flavonoid (Fluka); phenethyl ester (17% TIC) for esters (Bachem); and coumaric acid (6.1% TIC), caffeic acid (2.6% TIC), and benzoic acid (1.3% TIC) for aromatic acids (Fluka).[20] We generized a model propolis and a model wax composed out of the above-mentioned powdered substances to simulate the chemical composition of the main components of wax and propolis. For the model wax the substances were dissolved in chloroform, and the solvent was evaporated before recording the spectra. For the model propolis methanol was used as a solvent.

The comb samples were collected from *A. mellifera carnica* (Pollm.) colonies from the Zoological Institute at the University of Würzburg. The comb samples had not been used for storage of nectar and pollen or for brood keeping.

RESULTS AND DISCUSSION

Raman Mapping of Honeycombs

Figure 1 displays two representative Raman spectra of the standard wax of a honeycomb and of propolis. Both spectra exhibit significant features, which allow a clear distinction between both components. The marker bands that were selected for the Raman mapping diagram are indicated. We chose as marker bands for propolis the region from 1655 to 1570 cm^{-1} and for wax the region between 1080 and 1048 cm^{-1}. An important point for the selection of the marker regions is that they should not overlap with the spectral features of the spectrum to which they are compared.

Figure 2 shows the Raman mapping plots of a comb sample. Figure 2(a) shows the microscopic image of the investigated region of the sample. The box indicates the area were the *xy* Raman scan has been performed. The scanned region has a size of 20 $\times$ 36 μm. Points A and B show the positions where spectra A and B of Figure 3 were recorded. Figure 2(b) shows the false color plot of the intensity distribution of the wax marker band

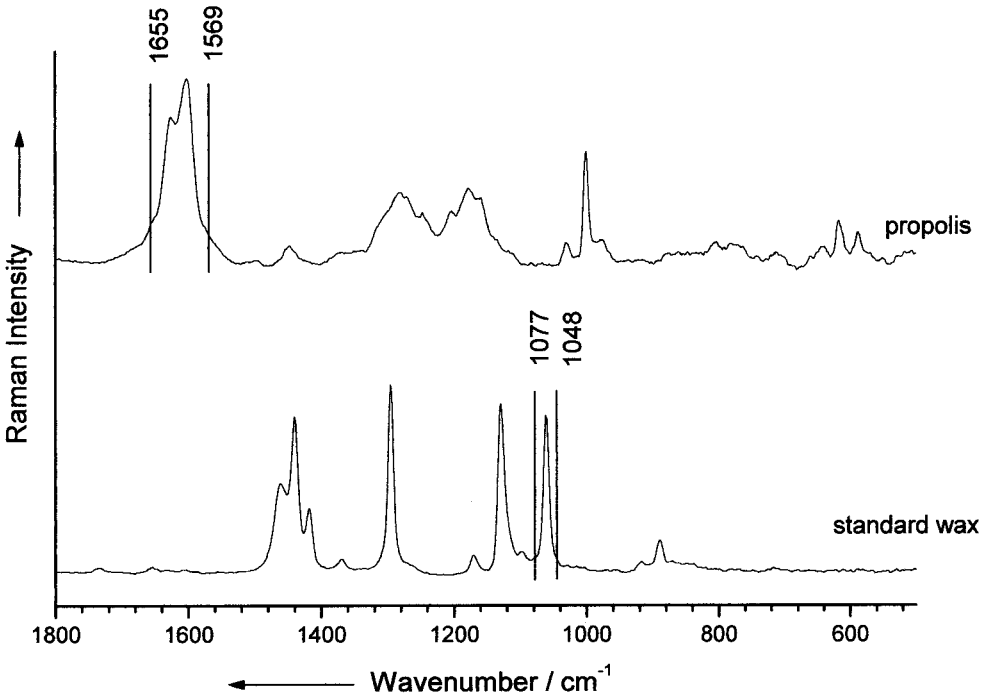

Figure 1. Raman spectra of propolis and wax of the honeybee *Apis mellifera carnic*a. The vertical lines show the marker bands of propolis and wax, which were used for the Raman mapping graphs (see Fig. 4).

and Figure 2(c) the intensity distribution of the propolis marker band. The brighter the color the higher is the intensity of the chosen band.

From Figure 2(a) there are different rough and dark or smooth and bright surface features that can be detected. A rough dark structure at the upper left part and a smooth light colored region in the lower right part of the image can be seen. However, these regions in Figure 2(a) cannot be assigned to a specific substance. On the Raman mapping plot in Figure 2(b) the distribution of the relative Raman intensity of the marker band of wax (1048–1077 cm^{-1}) is shown. Brighter colors indicate a higher intensity and therefore a higher concentration of the considered compound. Thus, the highest concentration of wax is in the upper left part of the plot where the rough dark parts can be seen in the microphotograph [Fig. 2(a)]. The lower right part shows a low wax concentration or no wax at all. Spectrum A in Figure 3 shows bands resulting from wax, as well as from propolis. This part of the sample ([Fig. 2(a), point A] contains a mixture of both substances.

Figure 2(c) shows the intensity distribution of the propolis marker band (1669–1655 cm^{-1}) as a false color plot, which shows the inverse situation as in Figure 2(b). The lower right part exhibits a high propolis concentration and the upper left part a low propolis concentration.

In Figure 3 two representative spectra of the 4500 spectra taken for the Raman images in Figure 2(a,b) are shown. It can be seen that spectrum A contains bands of the wax and propolis as indicated. This reveals that the two components are mixed in this region. This can also be seen in Figure 2(c). There are some regions in the plot that are not comletely dark blue (the Raman intensity is unequal zero), which indicates that there is some propolis in the upper left part of the sample as well.

Spectrum B in Figure 3 nearly contains only bands of propolis, which indicates that there is less wax in the lower right part of the sample. However, the bands at 1296, 1171, 1130, and 1064 cm^{-1} belong to wax, which indicates that there is also some wax in the lower right part of the sample. However, the intensity of these bands is low compared to the intensities of the propolis bands in this spectrum. In Figure 2(b) there are some regions where the color in the lower right part is gray, which indicates that there is a low intensity

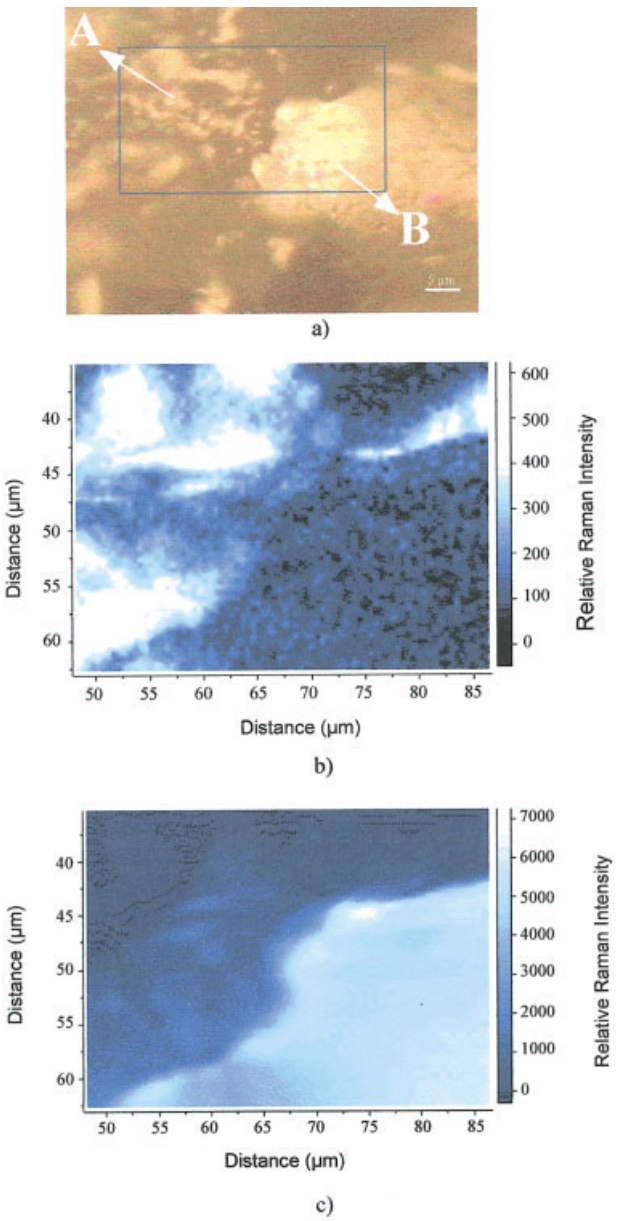

Figure 2. (a) A microphotograph of the area where the Raman mapping was taken. (b) The distribution intensity of the marker band of wax (1569–1655 cm^{-1}) over the scanned area shown in (a). A baseline correction was performed. (c) The distribution intensity of the marker band of propolis (1048–1077 cm^{-1}) over the scanned area shown in (a). A baseline correction was included.

of the marker band corresponding to a low amount of wax.

Because the Raman bands from wax and propolis can clearly be distinguished, it can be concluded that the two components are not mixed, instead remaining in two separate phases.

Raman Investigations of Wax and Propolis

After localizing propolis on different positions of the honeycomb, the question was still open of whether the propolis is used as collected or is modified by the honeybees. In order to answer this question of the use of this propolis, the chemical composition of wax and propolis was investigated by Raman spectroscopy.

The spectrum at the top of Figure 4 is one of natural bee wax (standard wax). For a tentative interpretation we tried to simulate the spectrum in two different ways. First, a spectrum of the calculated model wax was constructed by adding up the Raman spectra of the single standard component normalized to their percentage of occurrence (see Materials and Methods). A baseline correction (ninth-order polynomial) was carried out before the spectra were added.

Second, an artificially composed wax was mixed from the standard substances by dissolving the appropriate amounts of all components in chloroform, followed by drying the solution. (Further details are described in the Materials and Methods.)

The spectra of the standard components are displayed in Figure 4 together with the spectra of the standard wax, the composed model wax, and the calculated model wax. The wax spectra reveal a high degree of similarity, but they still have some discernible features. Although the intensity of the Raman bands at 1460 and 1440 cm^{-1} is identical in all three spectra, the intensity of the Raman mode at 1418 cm^{-1} is slightly changing. Its intensity is rising when comparing the calculated, model, and standard wax spectra. When comparing the spectra of standard and calculated model waxes, one can see that the ratio of the band at 1440 cm^{-1} compared to the bands at 1296, 1130, and 1062 cm^{-1} differs in these two spectra. The intensities of the bands at 1296, 1130, and 1062 cm^{-1} are much lower in the calculated model wax spectrum than in the composed model wax spectrum and the band at 889 cm^{-1} nearly disappears in the calculated wax spectrum. Therefore, the mixed model wax spectrum seems to be more similar to the natural standard wax spectrum than the calculated model one. We assume that this is the consequence of missing van der Waals interactions in the calculated wax spectrum. In order to mini-

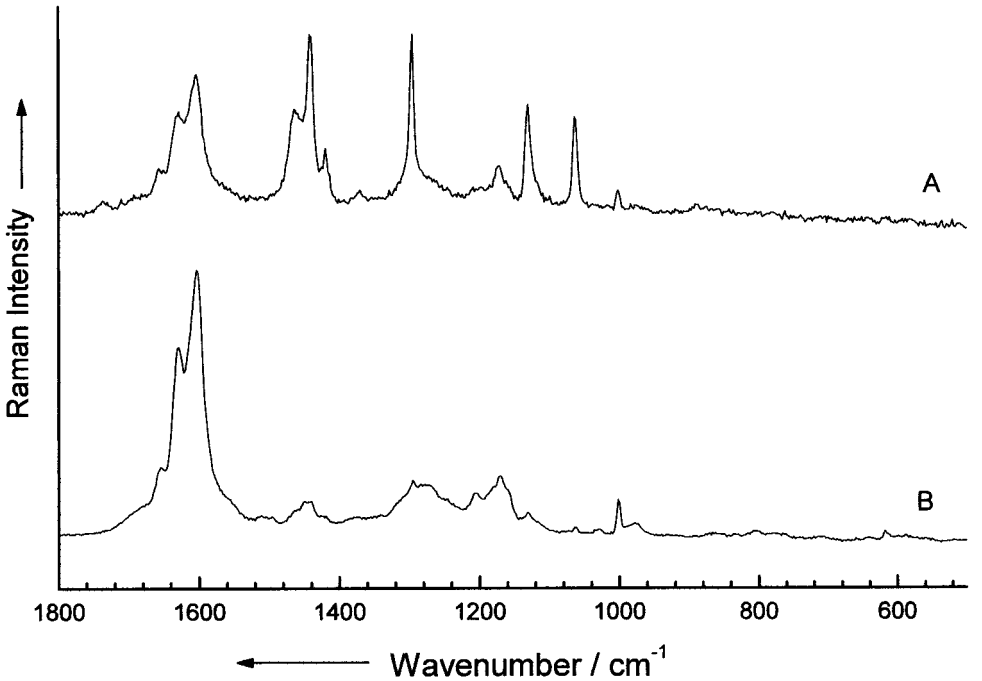

Figure 3. Raman spectra on points A and B in Figure 2. (a). The characteristic features for wax with additional content of propolis (spectrum A). The characteristic features for propolis with small peaks at 1296, 1171, 1130, and 1062 cm^{-1} that belong to wax (spectrum B).

mize a possible crystalline effect, the measurements were taken on different sample positions. All spectra were more or less identical.

The spectra of the standard substances are very similar. Raman bands of standard wax can be found in almost all spectra of the standard substances. The assignment of the observed bands is summarized in Table I.[21,22]

Figure 5 shows the results of the Raman spectroscopic investigation of propolis. The top spectrum of Figure 5 displays the one for natural propolis. The propolis sample was taken from a colony of *A. mellifera carnica* (Pollm.). To interpret these spectra we chose an approach analogous to the one we used to describe the Raman spectrum of the wax. A calculated model propolis was generated in an arithmetic procedure by adding up the Raman spectra of the single components with respect to their percentage of occurrence (see Materials and Methods). A baseline correction (ninth-order polynomial) was carried out before the spectra were added. The artificially composed model propolis was mixed out of the standard substances according to the procedure described in the Materials and Methods.

By comparing the spectra of the composed model propolis and the calculated model propolis one can see that the bands in the mixture overlap. This overlap might be caused by van der Waals interactions of the molecules in the mixture that are due to the different chemical environment of the molecules. One reason for this may be that not all of the components were involved in the analysis. In order to evaluate possible crystalline effects on the Raman spectra, different sample positions were measured. Again, no structural effects of the sample were observed.

The spectrum of the composed model propolis exhibits subtle differences in comparison to the spectrum of pure propolis. One can clearly see the bands at 1627 and 1603, 1001, 1360–1340, and 1150–1220 cm^{-1} are very similar to this spectral region in propolis. It can be observed that the propolis spectrum exhibits more spectral bands in the superposition spectra. The assignment of the observed Raman bands is summarized in Table II.[21,22]

An inspection of all spectra revealed that the positions and the intensities are different in some bands. The band at 1663 cm^{-1} in the calculated model propolis spectrum appears only as a shoulder in the natural propolis spectrum and can

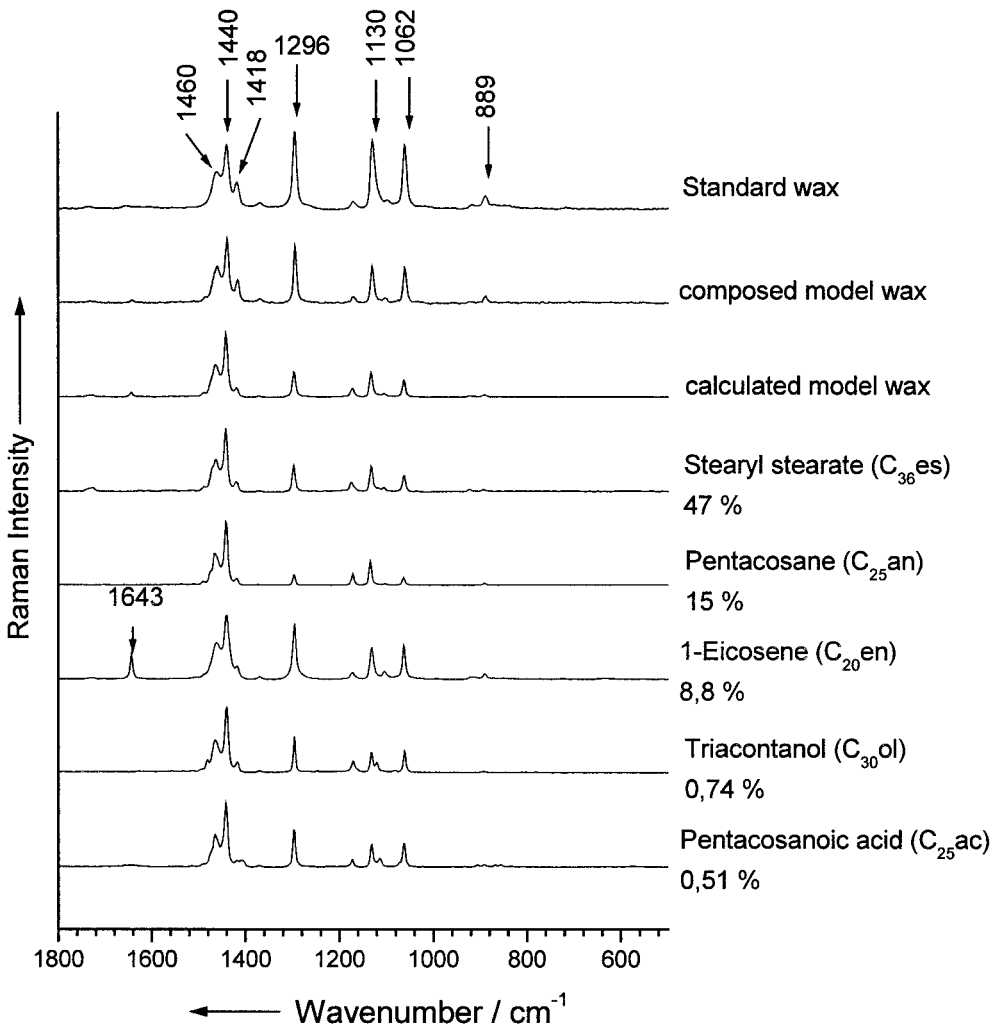

Figure 4. Raman spectra of standard wax and selected samples of the substance classes that represent the chemical composition of middle-aged wax.

hardly be seen in the spectrum of the composed model propolis. The relative intensity of the band at 1001 cm^{-1} is underestimated in the composed model and calculated model propolis spectra compared to the Raman spectrum of natural propolis. However, when taking into consideration that the standard substances used for these experiments were just a selection of the most abundant components, the overall agreement is very good.

Table I. Positions and Assignment of Raman Bands of Wax

Wavenumber (cm^{-1})	Assignment
1460	CH_3 scissoring mode
1440	CH_3 asymmetrical bending mode
1296	$(CH_2)_n$ inphase twisting mode
1232–885	C—C stretching modes
1643	Vibration of C═C double bond

The assignments are according to Lin-Vien et al.[21] and Dollish et al.[22]

Considering that neither the wax spectra nor the propolis spectra revealed changes in the band position and the band intensities, even though both can be detected at certain spots simultaneously, no obvious chemical changes of the substances were observed.

The Raman mapping plots have shown that propolis is located on the upper rim of the comb. Furthermore, in regions where the wax is located, the spectra show intense bands originating from pure propolis. For this it can be assumed that in a

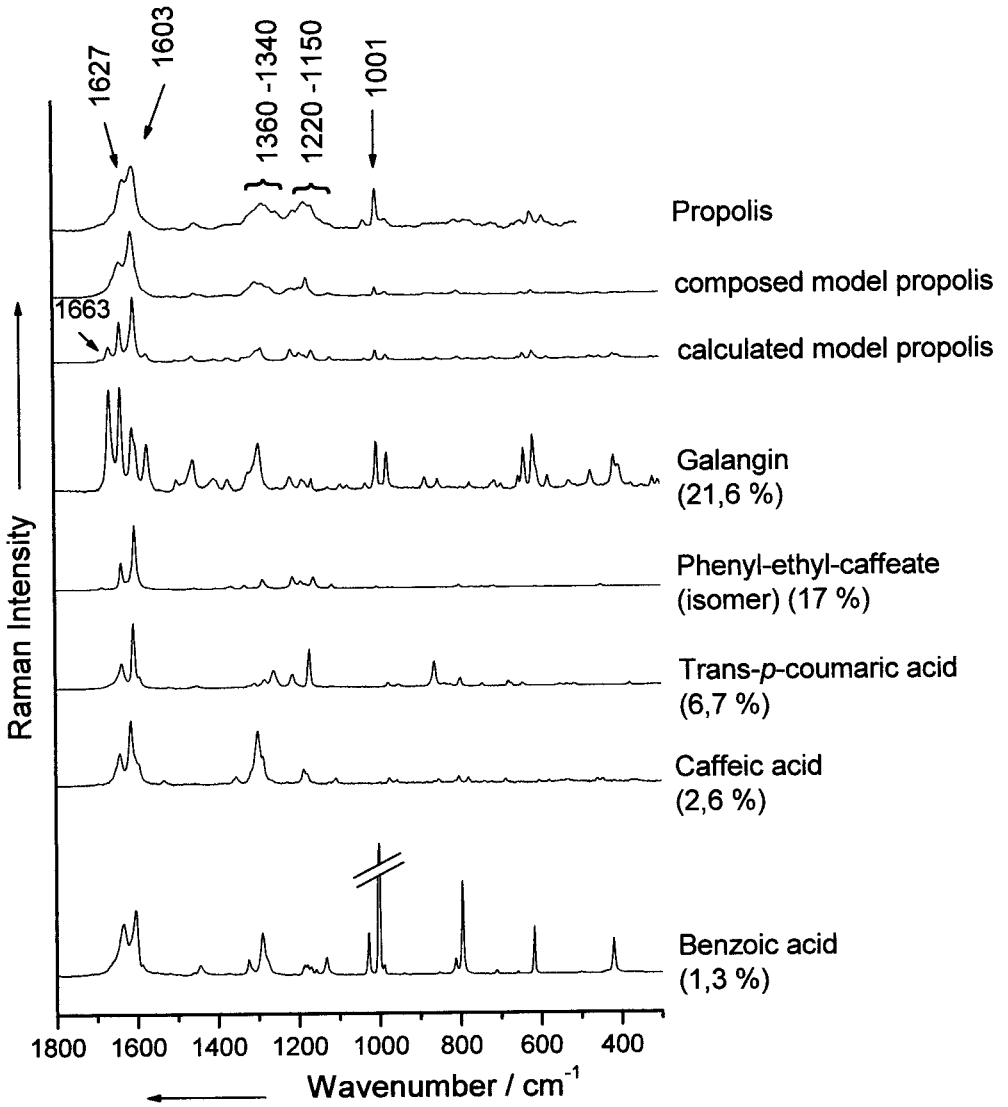

Figure 5. Raman spectra of propolis and selected samples of the compounds that are characteristic of the chemical composition of German propolis of *Apis mellifera carnic*a. For the addition, the spectra were normalized to the integration time and content (%).

Table II. Positions and Assignment of Raman Bands of Propolis

Wavenumber (cm^{-1})	Assignment
1627	Vinylic C═C double bond
1603	Aromatic C═C double bond
1001	Aromatic ring breathing

The assignments are according to Lin-Vien et al.[21] and Dollish et al.[22]

first step the bees build the comb out of pure wax and spread the propolis on the rims of the comb afterward. It seems that a first layer of wax is mixed with propolis to attach the propolis to the wax. The mixture consists of two phases. The propolis seems to be used more or less without any chemical modification by the honeybees. By further gluing propolis on this layer, a homogeneous thick layer of propolis arises. Spectrum B of Figure 3 shows almost no wax compounds (except the bands at 1296, 1171, 1130, and 1062 cm^{-1} with a very low Raman intensity), which indicates that there is almost no mixture of propolis and wax in this sample region.

The function of the propolis layer on the rims of the comb still remains to be elucidated. Darchen[7] and Chauvin[8] suggested that the propolis may act as a signal to stop in the construction of the comb cells. Other suggestions are that the purposeful application of this resinous substance can have several effects like influencing the melting behavior of the comb wax or stabilizing the comb. The stabilizing effect could play a role in the communication of the honeybees, which is a very interesting and fascinating scientific field in social biology. The use of propolis might clarify further observation of the bee's behavior while they carry the propolis into the hive.

CONCLUSIONS

In this work Raman spectroscopic investigations of the spatial distribution and chemical composition of wax and propolis in the comb of *A. mellifera carnica* (Pollm.) were performed. It was shown that propolis is used not only as a sealer for the hive and to disinfect the internal walls of the comb cells, but it is also found at the rim of the comb cells in a thick layer. The function of this rim remains to be elucidated. Propolis and wax are mixed at these rims to form a stable connection. No chemical modification of the two components was found.

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