Molecular structure of crude beeswax studied by solid-state ^{13}C NMR

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Abstract

^{13}C Solid-state NMR experiments were performed to investigate the structure of beeswax in the native state (crude beeswax) for the first time. From quantitative direct polarization ^{13}C MAS NMR spectrum, it was found that the fraction of internal-chain methylene (*int*-(CH_2)) component compared to other components of crude beeswax was over 95%. The line shape of the *int*-(CH_2) carbon resonance region was comprehensively analyzed in terms of NMR chemical shift. The ^{13}C broad peak component covering from 31 to 35ppm corresponds to *int*-(CH_2) carbons with *trans* conformation in crystalline domains, whereas the sharp signal at 30.3 ppm corresponds to *gauche* conformation in the non-crystalline domain. From peak deconvolution of the aliphatic region, it was found that over 85% of the *int*-(CH_2) has a crystal structure and several kinds of molecular packing for *int*-(CH_2), at least three, exist in the crystalline domain.

Keywords: Apis cerana japonica, crystallinity, conformation, chemical shift

Abbreviation:

NMR	nuclear magnetic resonance
int-(CH_2)	internal-chain methylene
CP	cross-polarization
MAS	magic angle spinning

Introduction

The waxes obtained from natural sources include animal waxes, vegetable waxes, mineral waxes, and petroleum waxes. Animal waxes are of insect or mammalian origin. The most important commercial animal waxes are beeswax and wool grease. Beeswax, with its unique characteristics, is now being used in the development of new products in various fields such as cosmetics, foods, pharmaceuticals, engineering and industry (Dorset 1999; Koga 2000; Mariya and Nikolay 2002; Al-Waili 2003).

Bees secrete wax from four pairs of special glands, called wax glands, on the underside of their abdomens. Beeswax consists of hydrocarbons, alcohols, free acids, and esters as well as other materials (Garnier 2002, Kimpe 2002, Tulloch 1972). Although the melting point of beeswax is about 60° C, it is interesting to note that wax is secreted in a liquid state at ambient temperatures. The liquid wax crystallizes in this condition. In general, the form of the crystal changes depending upon physical parameters such as temperature, pressure, and cooling rate. Therefore, it is probable that a specific structure is present in crude beeswax. However, only a few studies of the native structure of beeswax have been reported, especially of the Japanese bee, *Apis cerana japonica*. Structural and dynamic characterization of beeswax is necessary in order to understand the relationship between its properties and structure and, on the basis of these relationships, to design new applications for beeswax.

The investigation of beeswax by diffraction methods has had a long history (Dorset 1999; Dorset 1983). However, because the diffraction patterns from beeswax are not easily related to the well defined lamellar system crystallized from petroleum waxes, a significant amount of time has been spent resolving the X-ray diffraction and electron diffraction patterns of beeswax. Moreover, no information has been obtained on the amorphous or dynamic structures from X-ray diffraction analysis. Therefore, although valuable results have been obtained from diffraction analysis studies, it is considered that additional studies are necessary to resolve the structure of beeswax.

On the other hand, solid-state NMR spectroscopy is an important tool for the characterization of amorphous and semicrystalline solids (Kameda and Asakura 2003; Kameda et al. 2002a, Asakura et al. 1999) and has been applied extensively to probe the structures and dynamics in biopolymers (Kameda et al. 2003; Kameda et al. 2002b; Kameda et al. 1999a; Kameda et al. 1999b; Asakura et al. 2004; Asakura et al. 2001). Moreover, ^{13}C NMR should be able to provide information on dynamic aspects that cannot be obtained by diffraction methods. The ^{13}C chemical shift of solid materials is known to be sensitive to the crystal form

(Kameda et al. *in press*). Basson and Reynhardt (1988) demonstrated the ^{13}C CP/MAS spectrum for beeswax. However, no detailed structural analysis was carried out through ^{13}C chemical shifts, probably due to the poor separation of the peaks from the CH_2 region. Consequently, little progress has been made in trying to generate an understanding of the relationship between the structure of beeswax and its corresponding NMR chemical shifts.

Therefore, the purpose of the present work is to investigate the molecular structure of crude beeswax through the NMR chemical shifts of the internal CH_2 groups, in order to understand how the molecular structure of beeswax influence its amazing properties.

Materials and Methods

Materials

The sample of beeswax from the Japanese honeybee, *Apis cerana japonica,* was supplied by an apiarist (Mr. Seita Fujiwara) from Iwate prefecture in Japan. The sample of beeswax used in this study was in its native state (non-recrystallized). In general, impurities, such as pollen and honey, are part of natural beeswax. To obtain pure beeswax in its native state, the beeswax was taken before the bees could stock honey in the wax.

Solid-state NMR observations

Solid-state ^{13}C NMR spectra were obtained on a CMX 300 spectrometer (Chemagnetics, Fort Collins, CO, USA) at the Forestry and Forest Products Research Institute, Tsukuba, Japan, operating at a ^{13}C NMR frequency of 75.4 MHz. The samples were spun at the magic angle at 4 kHz in a solid-state probe in a 7.5-mm zirconia rotor (Chemagnetics). All spectra were obtained by using a ^{1}H NMR 90° pulse length of 5.0 μs and 60-kHz CW proton decoupling. ^{1}H-^{13}C CP contacts of 50kHz, and contact times from 0.01 and 8.0 ms were used. The repetition time for the CP experiments were 3.0s in all experiments. For the normal 90° single pulse sequence with high-power decoupling experiments (direct polarization ; DP), a 90° pulse width of 5.0μs and a repetition time of 1240s was employed. ^{13}C NMR spin-lattice relaxation time (T$_1$) was measured using the Torchia method. All spectra were calibrated by using adamantane as a standard as the CH_2 peak at 29.5 ppm gives shift values referenced to the TMS carbon at 0 ppm. Deconvolution of the NMR spectra was performed with an NMR peak simulator "ASA" produced by Dr. A. Asano (http://www.nda.ac.jp/cc/users/asanoa/NMR/progrm/index-e.html).

Results and Discussion

Peak assignments of CH_2 region

The ^{13}C MAS spectra obtained with direct polarization (DP) of native beeswax at room temperature are shown in Fig.1A. The assignment of the peaks was performed by comparison to references (Basson and Reynhardt 1988). The strongest resonance is centered between 30 and 35ppm, which are typical chemical shifts for internal-chain methylene (*int*-(CH$_2$)) carbons. The peak at 14.6ppm is due to methyl carbons at the terminus of alkyl chains. Figure1B shows expansions of the aliphatic region. In this spectrum, a 1240sec repetition time was used. The quantitative peak intensities were
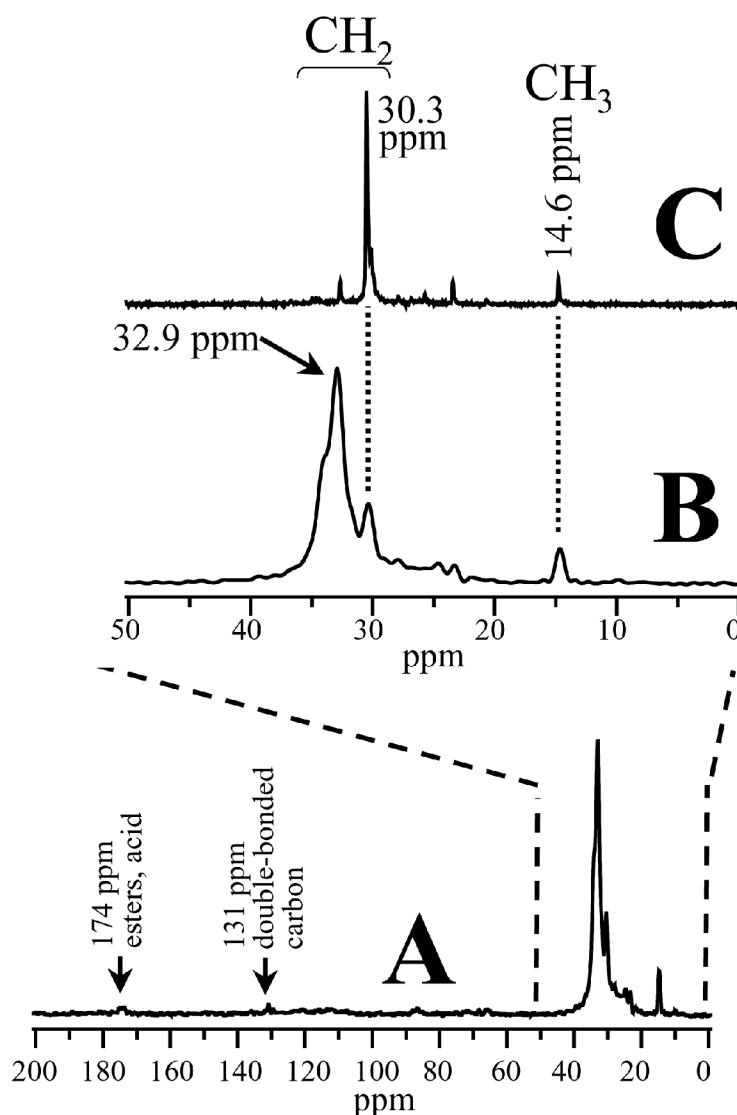

Figure 1. Full spectrum (A) and expansion of aliphatic region (B) at room temperature and melting temperature (C) of ^{13}C direct polarization NMR spectrum for the crude beeswax of Japanese honeybees (*Apis cerana japonica*).

compared in the DP spectrum whose intensities are accurate only if repetition time parameters allow sufficient time for complete relaxation of the ^{13}C resonance. From the comparison between Figs.2A and B, it was found that 97.1% of CH_2 carbon magnetization was recovered within 1240 sec. Therefore, setting the repetition time to 1240sec ensures that the relative intensities of the peaks are quantitative.

Although beeswax consists of hydrocarbons, alcohols, free acids, esters, and other materials (Garnier 2002, Kimpe 2002, Tulloch 1972), from Fig. 1A, it was found that the fraction of *int*-(CH$_2$) units with the other units is over 95%. This is because beeswax consists of long-chain carbon components including alkanes that contain 21–33 carbon atoms, acids that contain 22–30 carbons, and esters that contain 40–52carbons. Beeswax is also known to contain long-chain diesters (Tulloch 1972). Therefore, the structural study of beeswax mostly involves the structural elucidation of *int*-
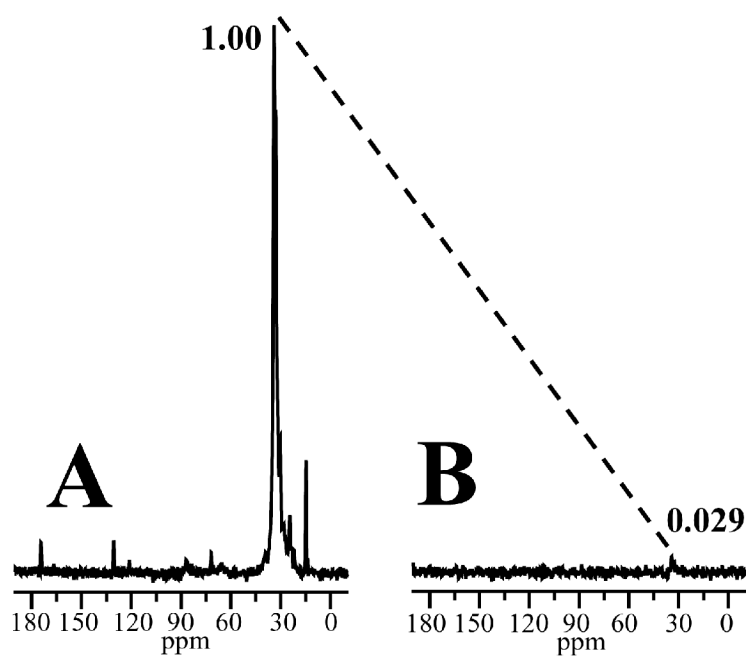
Figure 2. ^{13}C MAS spectra for the crude beeswax of Japanese honeybees obtained by Torchia pulse sequence with τ of 0 sec (A) and 1240 sec (B).

this investigation was very high. However, if the 32.9 ppm peak in Figure 1B is examined carefully, shoulder peaks can be seen on both sides of the main peak. The shoulder peak at 34.0 ppm was enhanced by using the CP method as shown in Figure 3 that shows expansion of the aliphatic region of the ^{13}C CP/MAS spectra with various contact times at room temperature. Such discrimination can be achieved between different signals if there are marked
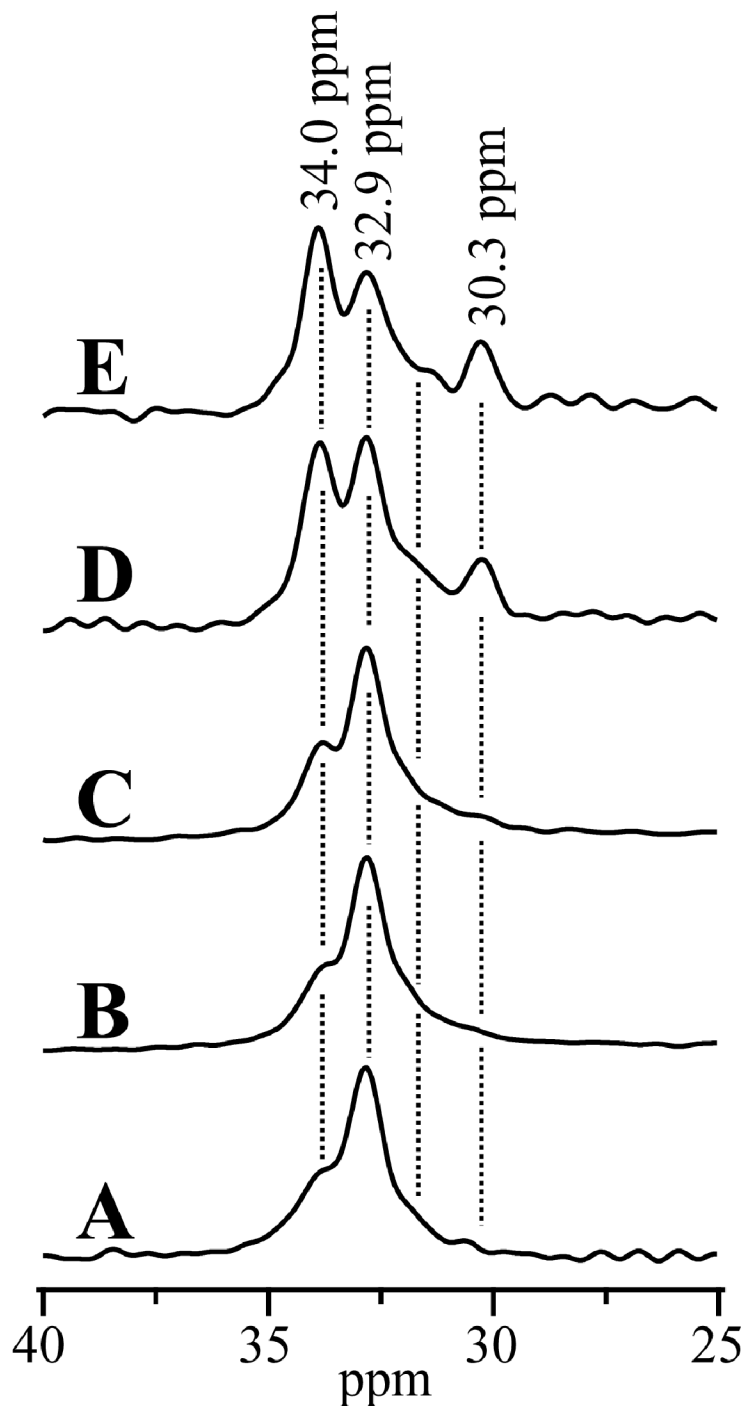
(CH_2) chains in the wax, although the proportion of hydrocarbon, alcohol, free acid, and ester components fluctuates greatly with the species and geographical habitat (Koga 2000). Thus, for the remainder for this paper, the focus will be on the *int*-(CH_2) region in the ^{13}C NMR spectrum.

Clearly, two separate signals at 30.3 and 32.9 ppm were observed for the CH_2 region in Fig.1B. At the melting temperature, the peak intensity at 30.3ppm increased, whereas the broad peak at 32.9 ppm was missing as shown in Fig.1C. Based on the fact that fast inter-conversion between *trans* and *gauche* conformations occurs at the melting point, it can be interpreted that the resonance of CH_2 carbon was shifted upfield due to the existence of *gauche* conformation, which is explained by the γ-effect (Tonelli and Schilling 1981). From this result, it can be interpreted that the peak at 30.3ppm in crude beeswax arises from the *int*-(CH_2) carbon in the *gauche* rich region. Similarly, in the case of *n*-alkanes, the CH_2 carbon with *gauche* conformation gives the peak around at 30.3 ppm (Ishikawa et al. 1991; Albert et al. 1998). In the solid-state, the CH_2 chains with *gauche* conformation exist in the non-crystalline region. Thus it was confirmed that the signal at 30.3 ppm in Fig.1B corresponds to the non-crystalline domain. In contrast, the ^{13}C broad peak component covering from 31 to 35 ppm with *trans* conformation in crystalline domains. These results demonstrate the utility of using a CH_2 chemical shift in understanding the semi-crystalline structure of beeswax.

Heterogeneous structure of crystal domain

Although some smaller peaks appeared (Fig.1C), the peak at 30.3 ppm had a high proportion of *int*-(CH_2), which indicates the purification of methylene compounds in the crude beeswax used in

Figure 3. ^{13}C CP/MAS spectra for the crude beeswax of Japanese honeybees with different contact times of 0.01ms (A), 0.1ms (B), 1ms (C), 5ms (D), 8ms (E).

variations in cross-polarization rates. Figure 4 shows the plots for the variation in ^{13}C peak intensities (arbitrary units) of *int*-(CH$_2$) peaks of 32.9 and 34.0 ppm with contact time at room temperature. Although the behaviors of the initial exponential rise in intensities at the shorter contact time for both peaks were remarkably similar, those of the exponential decrease in intensities at the longer contact time were different, which indicates that the T$_{1\rho}$ relaxation time for each *int*-(CH$_2$) carbon peak is different. From this result, it can be said that heterogeneity would exist in this broad peak, *i.e.*, the crystalline domain of beeswax consists of multiple components.

n-Alkanes are known to have various crystallographic forms such as orthorhombic, triclinic, monoclinic and hexagonal forms under certain conditions, in which the conformation is always the same all-*trans* zigzag. The main difference between these crystallographic forms is the orientation of the C-C-C plane in a *trans*-zigzag chain. The structure of *n*-alkanes has been successfully studied with solid-state high-resolution ^{13}C NMR spectroscopy. Previous studies have shown that the ^{13}C NMR chemical shift of *n*-paraffin depends on the crystal structure (Ishikawa et al. 1991). These influences of crystal structure on chemical shift are also theoretically explained by using an MO calculation (Yamanobe et al. 1985). Although the relationship between the crystal forms and its corresponding NMR chemical shifts for the beeswax is unclear, it can be said that several kinds of crystal forms exist in the crystal region of the beeswax.

Quantitative Analysis of gauche/trans ratio for crude beeswax

The quantitative peak intensities of *int*-(CH$_2$) carbons for crude beeswax were compared in the DP spectrum with a repetition

Figure 5. Deconvoluted ^{13}C direct polarization MAS spectrum of the internal-chain methylene region for the crude beeswax of Japanese honeybees.

time of 1240 sec (Fig.5). By deconvolution of the peak, it was found that, at least, four peaks at 30.3, 31.6, 32.9 and 34.0 ppm exist and the relative intensity for each peak was determined as 14.2, 4.5, 60.2 and 21.1%, respectively. From the intensity ratios of the crystalline peaks at 31.6, 32.9 and 34.0 ppm over the sum of the intensities of all CH$_2$ carbon peaks, the percentage of total amounts of the crystalline region was found to be 85.8%. If it is assumed that the ratio of the crystalline peak integral over the sum of the integrals for the crystalline and non-crystalline peaks of *int*-(CH$_2$) carbon correspond to the crystallinity of the beeswax, the crystallinities of the beeswax was determined to be over 85%. Although the degree of crystallinity would fluctuate to some degree with species and geographical habitat, it can be said that crude beeswax is a semi-crystalline material with high crystallinity and multi-crystal forms. The physical properties of beeswax should vary with the extent of crystallinity. Therefore, it would be worthwhile to measure quantitatively the degree of crystallinity of beeswax.

Conclusion

In this work, the solid-state NMR spectra of natural-beeswax in its native state from the Japanese honeybee, *Apis cerana japonica*, was observed for the first time. The chemical shift, cross-polarization rate, and T$_1$ relaxation data presented above provide a useful picture of the molecular structure of beeswax. Although
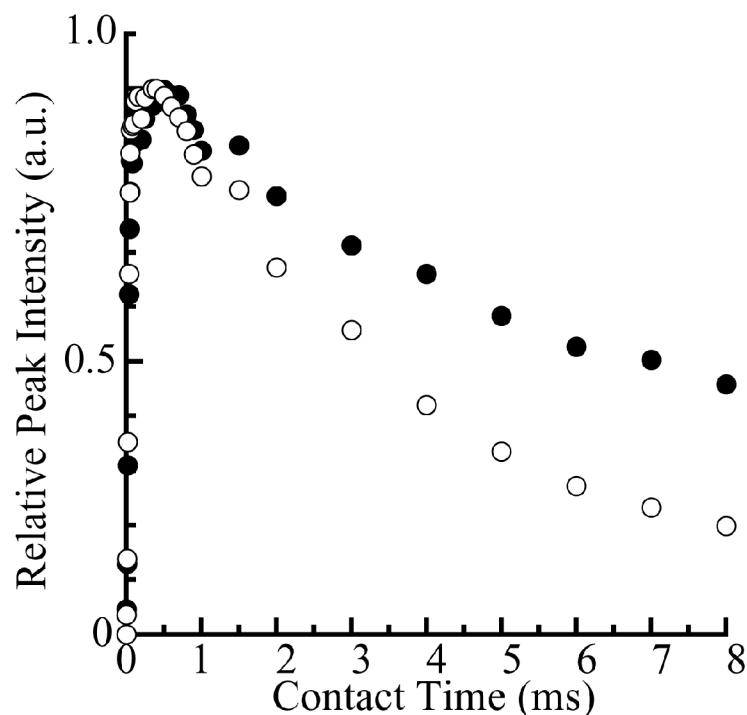

Figure 4. Contact time dependence of the internal-chain methylene carbons of 32.9 ppm (open symbols) and 34.0 ppm (solid symbols) at room temperature.
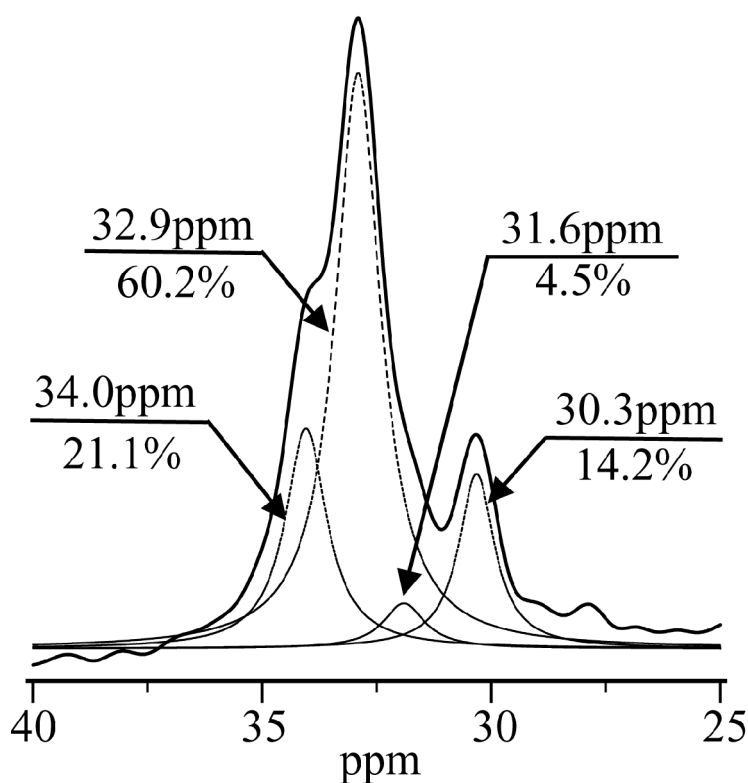
beeswax is known to be composed of multiple components, the fraction of methylene units compared to other units is over 95%. The ^{13}C chemical shift of the *int*-(CH$_2$) peak at 30.3 ppm reflects the *gauche* conformer. On the other hand, the broad peak at around 32.9 ppm was attributed to the presence of at least three components (34.0, 32.9 and 31.6 ppm) by curve fitting, indicating that there are at least three differences in the crystal packing in crude beeswax. The *int*-(CH$_2$) region of the DP spectrum has provided quantitative data on the crystallinity and the fraction of each crystal form. Finally, from these experimental findings, it was demonstrated that solid-state NMR spectroscopy is a useful means for elucidating the native structure of beeswax from honeybees.

Acknowledgements

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