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# Isolation and Characterization of Chitin and Biologically Active Substances from Honeybee (*Apis mellifera*)

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**Abstract**

The chemical composition of honeybees (*Apis mellifera*) was analyzed. Proteins accounted for the highest percentage of the detected substances (56.2%), followed by carbohydrates (11.3%), and lipid components (10.4 %). The mineral content was very low (0.5%). Thirteen fatty acids were identified, the main ones being oleic (56.0%), palmitic (22.6%), and stearic (11.6%) acid. Sterol composition was also determined with campesterol as a main component (50.2%). In addition, chitin was isolated from honeybee corpses by multistage chemical treatment involving deproteinization and demineralization. The chitin obtained (yield 8.8%) was characterized by physicochemical methods and Fourier transform infrared spectroscopy. On this basis, animal waste in the form of honeybee corpses was evaluated as a valuable source of chitin and other biologically active substances.

**Keywords:** honeybee (*Apis mellifera*), chitin, biologically active substances

## INTRODUCTION

Chitinous substances are among the most important renewable materials in the 21st century. They have multifunctional properties with applications in different fields such as the pharmaceutical industry, wastewater treatment, cosmetics, food additive production and agriculture [1, 2].

Numerous chitin sources can be found in the biosphere. With an annual turnover in the range of $10^{10}$ - $10^{12}$ tonnes, chitin is one of the most abundant biopolymers. Commercially, chitin and its derivatives are extracted from wastes of marine food production, mainly crustacean shells, e.g. shrimp, crab, crayfish or krill shells [3].

In recent years, some studies have emphasized that insects, mushrooms, coral and crustacean resting eggs can be alternative sources of chitin [1]. Domestic insects such as silkworm, typhoid fly and honeybee can provide considerable chitin biomass due to their fast reproduction [4].

As a result of the rapid development of beekeeping, significant attention has been paid to the honeybee (*Apis mellifera*) because of the opportunity to obtain larger amounts of material for chitin extraction and other biologically active substances. Some preparations derived from honeybees are used in the nontraditional or folk medicine [5]. Due to the common availability of bee corpses as a waste product of apiculture, they can be considered a new potential source of chitin and various biologically active substances.

Therefore, the specific object of this study was to isolate chitin and to determine the other chemical composition (lipid fraction, carbohydrates, protein, minerals and phenolic acids) of honeybee (*Apis mellifera*) corpses. Based on our results, the potential of honeybees as a nonconventional source for the isolation of chitin and bioactive compounds could be estimated.

## MATERIALS AND METHODS

**Animal material.** Honeybee corpses of local origin (Plovdiv region, Bulgaria) were obtained after hive cleaning in 2016. The animal material was cleaned of foreign particles, dried at 40 °C, finely ground in a laboratory mill to 0.5-1.0 mm particle size, and then stored in a desiccator for further use.

The moisture content of the animal material was determined by drying at 105 °C to constant weight [6].

Primary and secondary animal metabolites were analyzed in all samples and the values were represented on the basis of absolute dry weight.

**Lipid fraction.** The lipid fraction was extracted with n-hexane in a Soxhlet apparatus for 8 h. The solvent was partly removed by evaporation on a rotary vacuum evaporator. Then the residue was transferred to a pre-weighed glass vessel, and the rest of the solvent was removed under a stream of nitrogen to a constant weight in order to determine the oil content [7].

The total fatty acid composition of the lipids was determined using the GC-FID method after transmethylation of the respective sample with 2% $H_2SO_4$ in absolute $CH_3OH$ at 50 °C [8]. Fatty acid methyl esters (FAME) were purified by thin-layer chromatography on 20 cm x 20 cm plates covered with 0.2 mm Silica gel 60 G layer (Merck, Germany) with mobile phase n-hexane:diethyl ether at a ratio of 97:3 (v/v). Separation and determination was performed on a gas chromatograph equipped with a 75 m x 0.25 mm x 18 µm (I.D.) capillary Supelco column and a flame ionization detector (FID). The column temperature was programmed from 140 °C (5 min) at 4 °C/min to 240 °C (3 min). Injector and detector temperatures were set at 250 °C. Hydrogen was used as carrier gas at a flow rate of 0.8 mL/min; the split was 50:1. Identification was performed by comparison of the retention times with those of a standard mixture of FAME injected into GC under identical experimental conditions [9].

The unsaponifiable fraction was determined by weight after saponification of the lipids and extraction with n-hexane [10]. The unsaponifiable matter was applied on 20 cm x 20 cm glass plates (1 mm thick Silica gel G layer) and developed with n-hexane:diethyl ether at a ratio of 1:1 (v/v). Free sterols ($R_f = 0.4$) were detected under UV light by spraying the edges of each plate with 2´,7´-dichlorofluorescein, then the spots were scraped, transferred to small glass columns, and eluted with diethyl ether. The solvent was evaporated under a gentle stream of nitrogen, and the residue was weighed in small glass containers to a constant weight. Sterol composition was determined by GC using an HP 5890 gas chromatograph equipped with a 25 m x 0.25 mm DB-5 capillary column and a flame ionization detector. The temperature gradient rose from 90 °C (2 min) to 290 °C at a rate of 15 °C/min, and then up to 310 °C (10 min) at a rate of 4 °C/min; the injector temperature was 300 °C, and the detector temperature was 320 °C. Hydrogen was the carrier gas at a flow rate of 0.8 ml/min; split 50:1. The identification was confirmed by a comparison of the retention times with those of a standard sterol mixture [11].

The wax content in the lipid fraction was determined gravimetrically. For this purpose, 0.5000 g of extract was dissolved in $CH_3COCH_3$ at a ratio of 1:5 (w/v), then precipitated at 0 °C, filtered through a pad of *Celite* 545 and dried under vacuum at 50 °C to a constant weight.

**Biologically active substances.** Ultrasonic extraction of biologically active substances from honeybee corpses was carried out according to the method described by Petkova et al. [12]. The aqueous and ethanol extracts were filtered through filter paper and the resultant samples were used for further analysis.

**Carbohydrates analyses.** The total soluble carbohydrate content was estimated according to the method of Dubois et al. [13]. Briefly, 0.2 ml of the water extract were mixed with 1 ml of 5 % phenol, and 5 ml of concentrated $H_2SO_4$. The sample was left at room temperature (20 °C) for 30 min and after the reaction time the absorbance was measured at 490 nm against a control sample prepared in the same manner and containing distilled water instead of extract. The amount of soluble carbohydrates was determined from the calibration curve built with glucose as a standard ($y = 0.0098x - 0.0465$; $R^2 = 0.998$) [14].

Chromatographic analysis of the sugars presented in the extracts was performed on an ELITE LaChrome (Hitachi) liquid chromatograph equipped with a Sugar SP0810 (Shodex®) chromatographic column, Refractive Index Detector (VWR Hitachi Chromaster, 5450), and ELITE LaChrome (Hitachi) software. The elution was carried out in isocratic mode with a mobile phase of ultrapure water, flow rate of 0.5 ml/min, column temperature of 85 °C and 35 °C detector temperature. The volume of the injected sample was 20 µl.

**Phenolic acids.** Phenolic acids were analyzed on an ELITE LaChrome (Hitachi) liquid chromatograph equipped with a SUPELCO Discovery® HS C18 chromatographic column, diode-array detector (DAD) and ELITE LaChrome (Hitachi) software. The elution was carried out in gradient mode with mobile phase A: 2% $CH_3COOH$, and B: $CH_3CN$, at 25 °C. The volume of the injected sample was 20 µl.

**Protein.** Determination of the total protein content was carried out according to the Kjeldahl method described by AOAC [15]. The UDK 152 system (Velp Scientiffica, Italy) was used for the analysis. The sample (1.0000 g) was mineralized with concentrated $H_2SO_4$ (15 ml) and anhydrous $K_2SO_4$ and $CuSO_4$ as catalysts. The process was run at 420 °C for 60 min. This method used 40% NaOH to produce an alkaline distillation medium and 4% $H_3BO_3$ to collect the distilled ammonia. The titrations were carried out with a standard HCl (0.2 N) solution.

**Deproteinization.** Protein extraction was performed following the procedure of Marei et al. [16]. The dry skimmed bee meal was treated with 1 M HCl in a sample to reagent ratio of 1:50 (g/ml) for 12 h at ambient temperature (20 °C).

**Demineralization.** The removal of the mineral substances was performed in a dilute HCl solution according to the procedure of Marei et al. [16].

The demineralized solid fraction was filtered, washed with distilled water to neutrality and dried to a constant weight. The mineral substance content (%) in the meal was determined gravimetrically.

**Chitin characterization.** A commercial sample of chitin was obtained from Koch-Light Laboratories Ltd (England). The different physicochemical properties were measured as per the standard methods, e.g. moisture and ash content according to AOAC [17]. FTIR spectra were recorded in KBr tablets over a frequency ranging from 4000 to 400 $cm^{-1}$ at resolution of 4 $cm^{-1}$ using a spectrometer (Nicolet Avatar 330 FT-IR, Thermo Science, USA). The degree of acetylation (DA) of the bee and shrimp chitin samples was determined by comparing the absorbance of the measured peak to that of the reference peak at $A_{1655}/A_{3450}$. Therefore, the DA was calculated for chitin from the absorbance (A) ratios according to the following equation: $DA=(A_{1655}/A_{3450})\times115$.

**Statistics.** The measurements were performed in triplicate. The results are presented as mean value of the individual measurements with the corresponding standard deviation.

## RESULTS AND DISCUSSION

The chemical composition of honeybee has been shown in Table 1. The data indicated that protein accounted for the highest percentage of the detected substances, followed by carbohydrates and lipid components. The difference to 100% is explained by the presence of other substances in the raw material, such as melanin compounds, which are not defined in the present work [18] and are out of the scope of the current experiments.

Table 1. Chemical composition of honeybee corpses

| Components, % | |
|---|---|
| Moisture content | 10.7 ± 0.21 |
| Lipids | 10.4 ± 0.20 |
| Carbohydrates | 11.3 ± 0.14 |
| Proteins | 56.2 ± 0.36 |
| Minerals | 0.5 ± 0.01 |
| Chitin | 8.8 ± 0.20 |

The results obtained for protein and mineral content were lower than those published by Nemtsev et al. [18] for the same animal source: 50-80% and 2-3%, respectively. The differences can be explained by the origin of the bees and the methods of analysis used.

Three fractions of the honeybee with different polarity were examined: lipid, water, and ethanol.

The extracted lipid fraction presented a yellow-reddish semi-solid mass with a specific odor. The content of the specified components of the honeybee lipid extract is presented in Table 2. The data showed that unsaponifiable substances and waxes were predominant, and that was the reason for the fraction consistency. The amount of sterols and phospholipids was found to be very low.

Table 2. Content of the lipid fraction components

| Component, % | |
|---|---|
| Unsaponifiable substances | 28.3 ± 0.30 |
| Sterols | 3.1 ± 0.02 |
| Phospholipids | 1.4 ± 0.01 |
| Waxes | 24.9 ± 0.28 |

The fatty acid composition of the lipid fraction was determined. Thirteen fatty acids (saturated, mono- and polyunsaturated ones) were identified (Table 3).

Table 3. Fatty acid composition of the lipid fraction

| Fatty acids | | Content, % |
|---|---|---|
| $C_{8:0}$ | Caprylic | 0.2 ± 0.01 |
| $C_{10:0}$ | Capric | 0.2 ± 0.01 |
| $C_{12:0}$ | Lauric | 2.6 ± 0.05 |
| $C_{14:0}$ | Myristic | 1.0 ± 0.03 |
| $C_{15:0}$ | Pentadecanoic | 0.2 ± 0.01 |
| $C_{16:0}$ | Palmitic | 22.6 ± 0.40 |
| $C_{16:1}$ | Palmitoleic | 2.2 ± 0.08 |
| $C_{17:0}$ | Margaric | 0.5 ± 0.1 |
| $C_{18:0}$ | Stearic | 11.6 ± 0.23 |
| $C_{18:1}$ | Oleic | 56.0 ± 0.50 |
| $C_{18:2}$ | Linoleic | 1.3 ± 0.03 |
| $C_{20:0}$ | Arachidic | 0.8 ± 0.02 |
| $C_{22:0}$ | Behenic | 0.8 ± 0.02 |
| Saturated Fatty Acids | | 40.5 |
| Unsaturated Fatty Acids | | 59.5 |

Among them, monounsaturated oleic acid was predominant (56.0%), followed by saturated palmitic acid (22.6%) and stearic acid (11.6%). The amount of the other fatty acids varied from 0.2 to 2.6%.

It was obvious that monounsaturated fatty acids (58.2%) were predominant, followed by saturated fatty acids. Polyunsaturated fatty acids were presented only by linoleic acid in a relatively low quantity (1.3%).

The results obtained on the analyzed lipid fraction connected with the fatty acid content were in agreement with previous reports on other sources of animal origin [19] and some plant sources [19, 20, 21].

Sterols were presented and found in the unsaponifiable fraction of the lipids. Nine sterol compounds were identified, and their individual composition has been shown in Table 4.

Table 4. Sterol composition of the lipid fraction

| **Content, %** | |
|---|---|
| Cholesterol | 4.5 ± 0.10 |
| Brassicasterol | 2.2 ± 0.08 |
| Campesterol | 50.2 ± 0.60 |
| 24-Methylenecholesterol | 0.8 ± 0.02 |
| Stigmasterol | 9.7 ± 0.12 |
| $\Delta^7$-Campesterol | 11.7 ± 0.20 |
| β-Sitosterol | 16.5 ± 0.25 |
| $\Delta^5$-Avensterol | 3.2 ± 0.09 |
| $\Delta^7$-Stigmasterol | 1.2 ± 0.03 |

The results obtained demonstrated that the largest proportion of the detected sterols was due to campesterol (50.2%), followed by β-sitosterol (16.5%), $\Delta^7$-campesterol (11.7%) and stigmasterol (9.7%). The cholesterol amount in the sterol fraction was relatively high (4.5%). The sterol composition of the analyzed lipid fraction differed from that of other sources of animal origin [19].

Water and ethanol fractions were analyzed for free sugars and phenolic acids. The results from the chromatographic analysis of the skimmed honeybee meal have been summarized in Table 5. Only two monosaccharides, glucose and fructose, were identified in the aqueous extract, but only fructose was detected in the ethanol extract. This could be explained with the different solubility of sugars.

Out of the phenolic acids, only chlorogenic acid was found in the ethanol fraction. Gallic acid was detected in a trace amount in both fractions (water and ethanol extracts).

Table 5. Chemical composition of the aqueous and ethanol fractions

| Compound | Aqueous extract | Ethanol extract |
|---|---|---|
| | Sugars | |
| Glucose, mg/mg dry extract | 0.1 ± 0.00 | tr |
| Fructose, mg/mg dry extract | 0.9 ± 0.02 | 0.2 ± 0.00 |
| | Phenolic acids | |
| Gallic, μg/mg dry extract | tr | tr |
| Chlorogenic, μg/mg dry extract | tr | 0.1 ± 0.00 |

* trace - below 0.01%

After deproteinization and demineralization of the skimmed honeybee meal, chitin was obtained in an amount of 8.8%, ± 0.20, which does not contradict the data published by Nemtsev et al. [18] for the same source material (10-12%).

Some physicochemical characteristics of chitin isolated from honeybee corpses were evaluated and presented in Table 6. The listed information did not show significant differences compared to the commercial chitin sample.

Table 6. Physicochemical characteristics of the isolated and commercial chitin samples

| Characteristic | Isolated chitin | Commercial chitin |
|---|---|---|
| Appearance | flaky substance | |
| Color | beige to light brown | light brown |
| Odor | odorless | |
| Moisture content, % | 7.7 ± 0.09 | 6.4 ± 0.07 |
| Ash, % | 2.4 ± 0.03 | 0.1 ± 0.00 |

The isolated chitin was characterized by a darker color and higher ash content in comparison with commercial chitin. The darker color was probably due to a residual melanin content that cannot be completely eliminated by deproteinization with a dilute alkaline solution. Some authors recommend the use of sodium hypochlorite solution for chitin bleaching [5, 22].

The information published in the scientific literature in connection with the physicochemical characteristics of chitin varies within a wide range. Hossain and Iqbal [23] reported chitin moisture and ash content of 8.5-9.23% and 0.36-4.24%, respectively. However, Paul et al. [24] and Ibitoye et al. [25] reported 4% moisture content in chitin and ash content of 1-1.86%. In the research work of Abdulkarim et al. [26], higher values of dry matter and ash content were established: 12.9% and 26.45%, respectively. In previous reports, the chitin yield of the house cricket was 4.3-7.1%, which was close to our chitin yield from bees. The chitin content of bees in this study was comparable to those of microcrustaceans and some insects as in Daphnia (3-7%), of grasshoppers (5.3%-8.9%), beetles (5%) and the spider species *Geolycosa vultuosa* (8-8.5%) and *Hogna radiate* (6.5-7%) [25].

The differences in dry matter are most likely due to insufficient drying and to storage conditions. Ash content is an indication of residual mineral content in the final product, and the data differences probably result from an ineffective demineralization process.

Various adsorption bands within the 4000 – 400 $cm^{-1}$ range were recorded in the FTIR spectra of chitin isolated from honeybee. These bands were compared with those of the commercial sample. The data in Figure 1 and Table 7 indicate that the adsorption bands of the extracted and commercial chitin were identical.

Comparable results on the isolation and characterization of chitin from marine sources in the Black Sea (*Liocarcinus vernalis* and *Caridea*) were published by Zvezdova and Stoeva [27]. Similar data from chitin spectral analysis were also published by Kaya et al. [1], who isolated chitin from two spider species (*Geolycosa vultuosa* and *Hogna radiata*).

Infrared spectroscopy was used to characterize the chitin isolated from honeybee (Figure 1). The FTIR spectra of the commercial chitin and the honeybee chitin showed similar bands (Table 1). In particular, OH stretching band at 3442 $cm^{-1}$, N-H stretching band at 3268 $cm^{-1}$, amide band I at 1660 and 1621 $cm^{-1}$, and amide II band at 1558 $cm^{-1}$ were observed. These absorption peaks are especially characteristic of chitin [26, 28-30]. Bands that are related to secondary amides, to chitin in particular, were presented in the FTIR spectra: 3443–3269–3107–1661–1558–1315–1261 $cm^{-1}$. The FTIR spectrum of chitin isolated from honeybee corpses possessed typical signals at 1650, 1550 and 952 $cm^{-1}$, which were assigned with the amide I, II and III bands, respectively. Another absorption band appeared at 1656 and near

1420 $cm^{-1}$ corresponding to the (amide I) stretching of the C=O bonds of the acetamide groups and symmetric deformation of $CH_3$. The band at 1558 $cm^{-1}$ corresponded to the N–H deformation of amino group (amine II). A sharp signal appeared at 1379 $cm^{-1}$, caused by the $CH_3$ symmetrical deformation. The peaks observed at 1070 and 1029 $cm^{-1}$ were the secondary hydroxyl group (characteristic peak of –CH–OH in cyclic alcohol, C–O stretch) and the primary hydroxyl group (characteristic peak of $–CH_2–OH$ in primary alcohol, C–O stretch), respectively. The absorption band at 1157 $cm^{-1}$ was the asymmetric stretching of the C–O–C linkage. The bands were in accordance with previous reports for chitin obtained from different animal sources [27, 29-31].

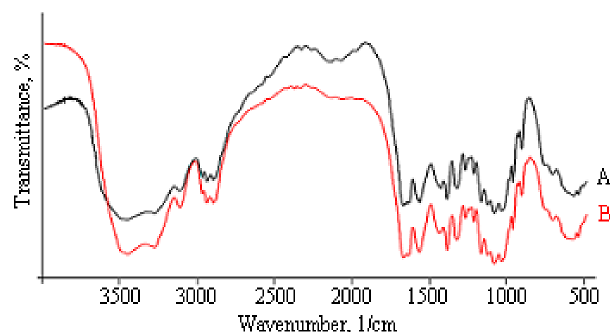


Figure 1. FTIR spectra of commercial (A) and isolated (B) chitin

Table 7. FTIR characteristic adsorption bands of commercial chitin and chitin isolated from honeybee

| Band frequency ($cm^{-1}$) | | Assignments [26, 28-32] |
|---|---|---|
| Isolated | Commercial | |
| 3442 | 3446 | ν(O-H) – in the pyranose ring<br>ν(N-H) – non assos. in secondary amides<br>ν($NH_2$) – assos. in primary amines |
| 3269<br>3107 | 3269<br>3107 | ν(N-H) – assos. in trans-secondary amides<br>ν(N-H) – assos. in cis- and trans-secondary amides |
| 2963 | 2961 | $ν_{as}(CH_3)$ in $NHCOCH_3$ group |
| 2933 | 2931 | $ν_{as}(CH_2)$ in $CH_2OH$ group |
| 2891 | 2891 | ν(C-H) in the pyranose ring |
| 1661 | 1661 | ν(C=O) in $NHCOCH_3$ group (amide I), N-acetyl group in chitin |
| 1558 | 1556 | Complex vibration δ(N-H) + ν(C-N) in trans-secondary amides (amide II) |
| 1431 | 1418 | δ($CH_2$-OH) |
| 1379 | 1378 | $δ_S(CH_3)$ in $NHCOCH_3$ group |
| 1315 | 1315 | δ(C-H) in the pyranose ring; C–N stretching in<br>secondary amide (amide III) |
| 1261;<br>1205 | 1261; 1205 | Complex vibration of NHCO group (amide III band in secondary amides only) |
| 1157 | 1157 | $ν_S$(C-O-C) – glycosidic linkage |
| 1116.6 | 1115 | $ν_{as}$(C-O-C) – glycosidic linkage |
| 1074.2 | 1074 | ν(C-OH) – in secondary OH group |
| 1028 | 1028 | ν(C-OH) – in primary OH group |
| 953 | 953 | $CH_3$ wagging, amide III |
| 896 | 896 | C-anomeric groups stretch, C1–H-deformation; pyranose ring stretch; β-anomer of glucopyranose |

In the present study, the DA value of 96.1% was calculated for chitin extracted from honeybee. However, it is slightly higher than the value previously reported for other shrimp (94.3%) and *Holotrichia parallela* (93.1%), determined using the same formula [26]. Nevertheless, our results are in good agreement with DA for chitin from other insects such as locusts (98%), honeybees (96%) and beetles (95%) [16]. In addition, DA values of about 90% are considered typical of chitin [30].

## CONCLUSIONS

After suitable chemical treatment, honeybees (*Apis mellifera*) could be used as an alternative source of chitin (8.8%) and other biologically active substances such as proteins (56.2%), lipids (10.4%) and carbohydrates (11.3%). According to the analyses conducted, the lipid extract contained unsaponifiable substances (28.3%), sterols (3.1%), phospholipids (1.4%), and waxes (24.9%). Thirteen fatty acids were identified, the main ones being oleic (56.0%), palmitic (22.6%) and stearic (11.6%) acid. Campesterol predominated in the sterol fraction of the lipids. The results of the HPLC analysis obtained from the skimmed honeybee meal showed that two monosaccharides, i.e. glucose (0.1 mg/mg extract) and fructose (0.9 mg/mg extract), were identified in the aqueous extract, and only fructose (0.2 mg/mg extract) in the ethanol extract. Chlorogenic acid (0.1 μg/mg extract) was found only in the alcohol fraction, and gallic was found in traces in both fractions. Based on the results, honeybees can be viewed as a potential nonconventional source for the isolation of chitin and bioactive compounds with applications in cosmetics and pharmaceutical products.

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