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The Extraction and Characterization of Chitosan from *Apis Mellifera*

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Abstract Currently, numerous studies are being done to identify and use new sources of chitin. For this purpose, in our research, we aimed to obtain chitosan-based and local raw material *Apis mellifera* and analyze its structure through FTIR, NMR, and XRD methods. In our study, we initially isolated α chitin and chitosan with an amorphous crystallinity of 31.65%.

Keywords Chitin, Chitosan, α–chitin, Crystalline index, FTIR spectroscopy, ^{1}H and ^{13}C NMR, XRD (X-ray Diffraction)

1. Introduction

Recently, the demand for products made from natural polysaccharides (polymers) is growing. They have many application fields, due to their biodegradable, biocompatible, anticancer, antibacterial, antifungal activities, etc. Not only are natural polysaccharides but also their biosynthetic polymers are biodegradable and eco-friendly. These types of polysaccharides include alginate, carrageenan, agarose, glucomannan, galactomannan, pectin, chitin and chitosan, hyaluronic acid derivatives, and others. The various biological functions of natural polysaccharides are their most important properties [1]. Interest in chitin and chitosan polyaminosaccharides is growing since their non-toxic, biocompatible, biodegradable, possession of many therapeutic properties (antioxidant, antimicrobial, antifungal, high activity against tumor and cancer cells, analgesic, hemostatic, etc.) for use in the biomedicine, pharmacy, cosmetology, and food industry [2,3]. In nature, chitin occurs in a variety of organisms, including infusoria, amoebae, chrysophytes, some algae, fungi, crustaceans, worms, insects, mollusks, and in the cell walls of fungi and microorganisms (bacteria) [4,5], while chitosan is found in zygomycetes and mucosa, as well as in some fungi such as Absidia coerulea [6]. Globally, chitin and chitosan are mainly isolated from crustaceans, and many studies have recently been carried out to obtain them from insect cuticles [7]. While the use of these types of raw materials on an industrial scale has some disadvantages, some types of insects can be used to extract large amounts of chitin. These are insects that can be reared artificially (honeybees, silkworms, and synanthropic flies). The chitin substance found in insect cuticles is covalently bound to melanin and sclerotin-like proteins [8], which can cause some problems in the process of chitin extraction. The wide development of beekeeping in our country, allows us to use bees (Apis mellifera) as a source of chitin. For this purpose, we obtained chitosan from Apis mellifera first by extracting chitin and then by deacetylation of chitin and used physical research methods (FTIR, 1H and 13C NMR, XRD) to study its structure. We analyzed the structure using spectroscopy methods and crystal structure through the X-ray diffraction method.

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2. Materials and Methods

2.1. Extraction of Chitin from *Apis mellifera*

A certain amount of dried and crushed *Apis mellifera* was weighed and to remove impurities, heated with distilled water at 70-80℃ while 1 hour. After that resulting material is filtered and dried in an oven at 60℃ for 24 hours. This dried material was refluxed with an aqueous solution of 0.1 N HCl at 70-80℃ for 1 hour. The demineralization process was completed by washing in a neutral medium with deionized distilled water. Then dried in an oven at 60℃ for 24 hours. To achieve the deproteinization process resulting dried material was refluxed with an aqueous solution of 0.1 N NaOH at 70-80℃ for 4 hours and filtered, washed to a neutral medium, and dried. For the decoloration and bleaching process, the resulting material was stored in a 30% H_2O_2 solution for 24 hours. Then extracted chitin plates were washed and dried in an oven at 60℃ for 24 hours.

2.2. Deacetylation of Chitin

Extracted chitin was refluxed with a 40% aqueous solution of NaOH at 70-80℃ for 10 hours. Then obtained chitosan (deacetylated chitin) is filtered and washed until neutral medium and dried in an oven at 60℃ for 24 hours. The general process of chitosan extraction is depicted in the following Fig. 1:

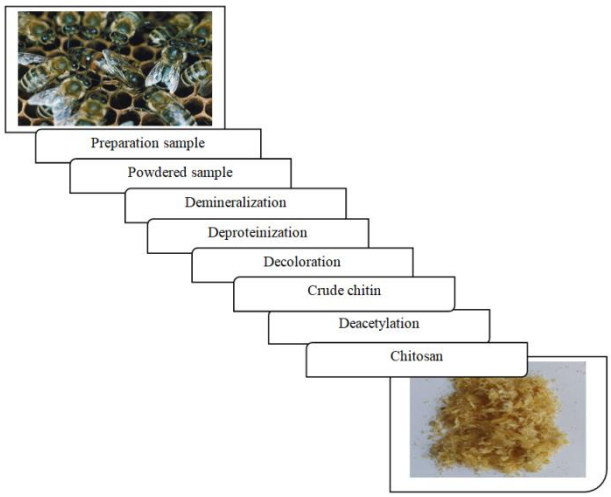

Figure 1. The general process for chitosan extraction

2.3. Fourier Transform Infrared Spectroscopy (FTIR)

Chitin and chitosan obtained from *Apis mellifera* were characterized from 4000-650 cm^{-1} by a Perkin-Elmer FTIR spectrophotometer.

X-ray Diffraction (XRD)

XRD patterns were measured with a Shimadzu X-ray diffractometer (XRD-6100) with CuKα1 – α2 (Kα1 α2 ratio 50%) radiation (γ= 0.15406 nm, 40 kV, 30 mA, scan range 10° - 80°, scanning rate 2°/min and step length 0.05°. The crystalline index (CrI) was calculated by the following equation [12]:

$$Crystalline\ index(x) = \frac{(I110 - Iam)}{I110} x100\% \qquad (1)$$

where I_{110} = the maximum intensity at 2θ = ∼20° and

I_{am} = the intensity of amorphous diffraction at 2θ = ∼16°;

^{1}H and ^{13}C Nuclear Magnetic Resonance (NMR)

NMR spectra of extracted chitosan were recorded on a JNM-ECZ600R spectrometer (600 MHz for 1H and 150 MHz for 13C, Jeol, Japan) at 303 K in 1%CD3COOD in D2O as a solvent with a standard parameter set. The proton and carbon signals of CD3COOD (2.08 and 21.03 ppm vs. TMS respectively) were used as a reference for 1H and 13C NMR shifts.

3. Results and Discussion

In our study, first chitin and then chitosan were isolated based on *Apis mellifera*, and the obtained substances were analyzed by FTIR spectroscopy, 1H, and 13C NMR, and the X-ray diffraction methods. The following are the results obtained through these methods.

3.1. FTIR Analysis

The IR spectrum of chitin obtained from *Apis mellifera* is shown in Fig. 2. According to the literature survey, the amide I band of α chitin consists of two peaks [8]. The fact that the extracted chitin amide I band consists of two peaks at 1652 and 1620.61 cm^{-1} indicates that the extracted chitin is in the α form.

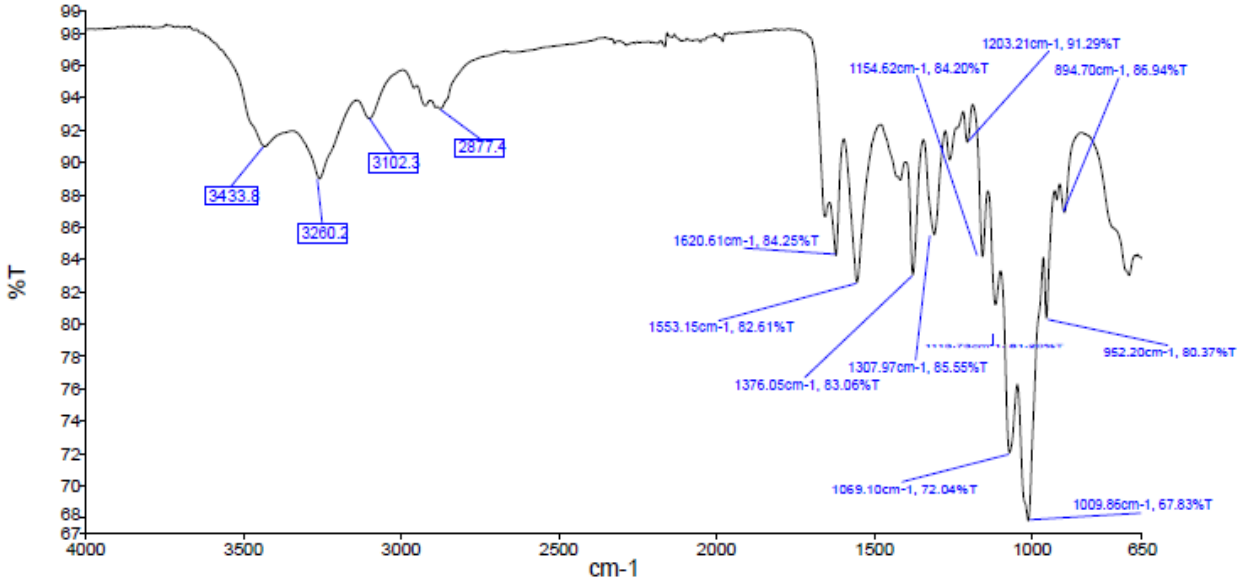

Figure 2. FTIR spectra of extracted chitin

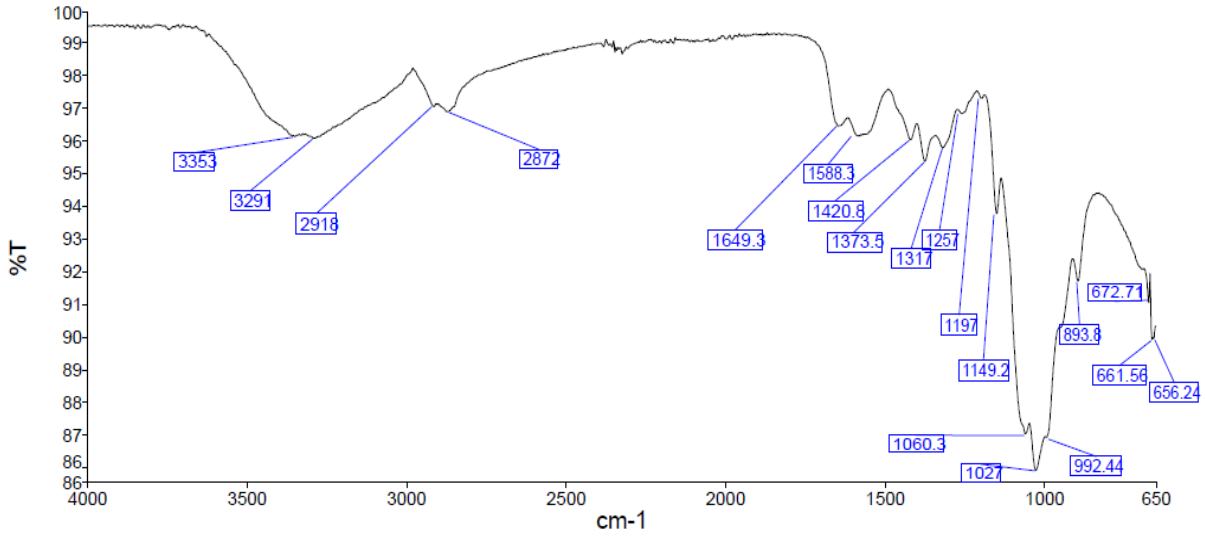

Figure 3. FTIR spectra of extracted chitosan

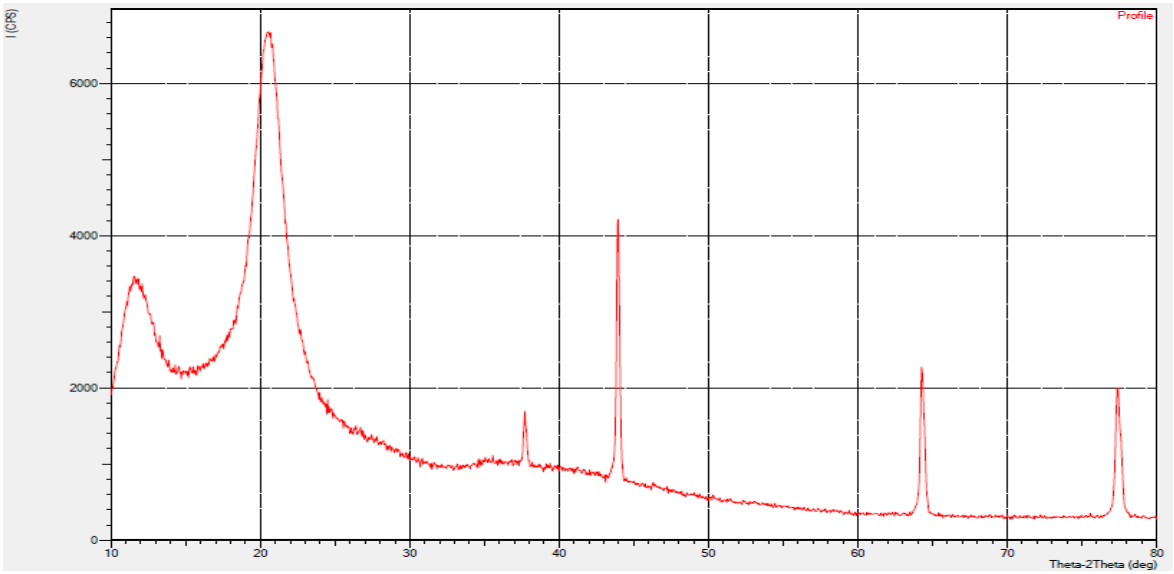

Figure 4. Label XRD peak of extracted chitosan

The study of the IR spectrum of extracted chitin also showed the following signals: 3433 cm^{-1} (O-H stretching), 3102.3-3260.2 cm^{-1} (N-H stretching), 2877.4 cm^{-1} (methylene groups in the pyranose ring); 1553.15 cm^{-1} (amid II), 1430 cm^{-1} (CH2 bending and CH3 deformation), 1307.97 cm^{-1} (CH2 wagging), 1154.62 cm^{-1} (asymmetric bridge oxygen stretching), 1115 cm^{-1} (asymmetric in-phase ring stretching mode), 1069.10 cm^{-1} (saccharide rings), 1009.86 cm^{-1} (C – O asym. stretch in phase ring), 952.20 cm^{-1} (along chain) and 894.70 cm cm^{-1} (saccharide rings). The changes that occurred in the signals indicate a decrease in the length and molecular weight of the polymer chain [9,10].

The main changes in the IR spectrum of chitosan obtained through the chitin deacetylation occur in the 1650 and 1590 cm^{-1} regions. The appeared and expanded signal at the 1590 cm^{-1} band, and the decrease in intensity at the 1650 cm^{-1} band indicates deacetylation quality and chitosan formation [9,11]. IR spectrum of the extracted chitosan signals was observed in the 1649.3 and 1588.3 cm^{-1} regions, and these changes are similar to the data presented in the literature. Also, changes in the intensity of absorption signals at the 900-1100 cm^{-1} band indicate a decrease in the length and molecular weight of the polymer chain within deacetylation [9]. In the FTIR spectrum of extracted chitosan from *Apis mellifera*, signals were also observed in the following areas: 3353, 3291, 2918, 2872, 1649.3, 1588.3, 1420.8, 1373.5, 1317, 1257, 1197, 1149.2, 1060.3, 1027, 992.44, 893.8, 672.71, 661.56 and 656.24 cm^{-1} (signals that the main change occurs).

3.2. XRD Analysis

The XRD method was used to study the crystalline structure (crystallinity index and crystalline lattice) of chitosan obtained based on *Apis mellifera* (Fig. 4).

At 2θ = 10° and 20°, semi-crystalline chitosan forms broad diffraction peaks that are characteristic fingerprint areas [12], which peaks observed at 12° and 20.57° in the *Apis mellifera*

chitosan. Characteristic peaks in this area indicate free amino groups of chitosan form intermolecular and intramolecular hydrogen bonds [13]. However, the broad diffraction peaks of chitosan extracted from *Apis mellifera* relative to standard chitosan are related to its amorphous nature [14]. The formation of different characteristic peaks may also vary depending on the type of chitin source [15]. The XRD results of chitosan obtained based on *Apis mellifera* are given in Table 1 below. As can be seen from Table 1 above, the crystalline index of extracted chitosan based on *Apis mellifera* is 31.65% and the crystalline lattice has a tetragonal structure.

3.3. ^{1}H NMR Analysis

The structure (Fig. 4.) and the 1H NMR spectrum of the extracted chitosan are depicted in Fig. 5. The signal at 2.0-2.1 ppm represents the three protons of N-acetyl glucosamine (GlcNAc) and the signal at 3.1-3.2 ppm represents the H-2 protons in the glucosamine (GlcN) residue. The non-anomeric protons bound to C3 – C6 in the glucopyranose ring have the same chemical shift values because they have the same electron densities. Because the non-anomeric protons in the spectrum of the molecule are partially overlapped, the signals between the spectra are in the expanded state, and all signals are observed between 3.5 and 4 ppm.

Anomer protons (H1) are observed at higher chemical shift values. This is due to their neighboring glycoside and ring oxygen. The protons of H-1 [GlcN (H-1D) and GlcNAc (H-1A)] signal at 4.6 and 4.8 ppm. Among the different regions of the 1H NMR spectrum, methyl protons give a signal at 2.0–2.1 ppm, which is the highest resolution. The protons of H-1 [H1 (GlcNAc) 4.8 and H1 (GlcN) 4.6 ppm] have the least resolution. Protons H3, H4, H5, and H6 also have low resolution. The signals of the last protons overlap with the HOD signals of the solvent (D2O / CD3COOD) at 4.05 ppm.

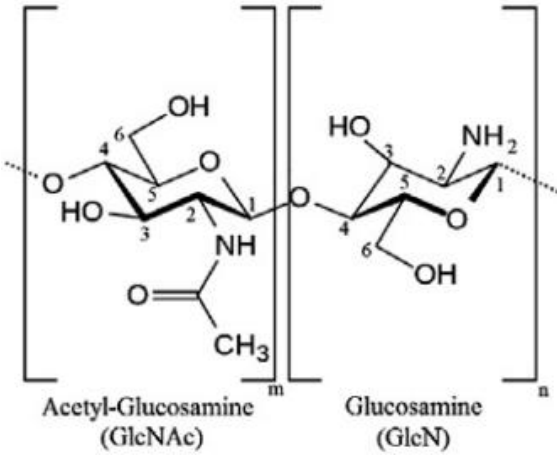

Figure 5. Structure of chitosan

Table 1. XRD peak data list of extracted chitosan

Sample name	Peak no.	2 Theta (deg.)	d (spacer value) (A°)	I/I1 relative intensity (%)	Crystal-line index	Crystal system
Chitosan		12.008	7.364	29	31.65%	Tetragonal
		20.571	4.314	100		
		37.692	2.384	6		
		43.922	2.059	19		
		64.263	1.448	12		
		77.372	1.232	14		

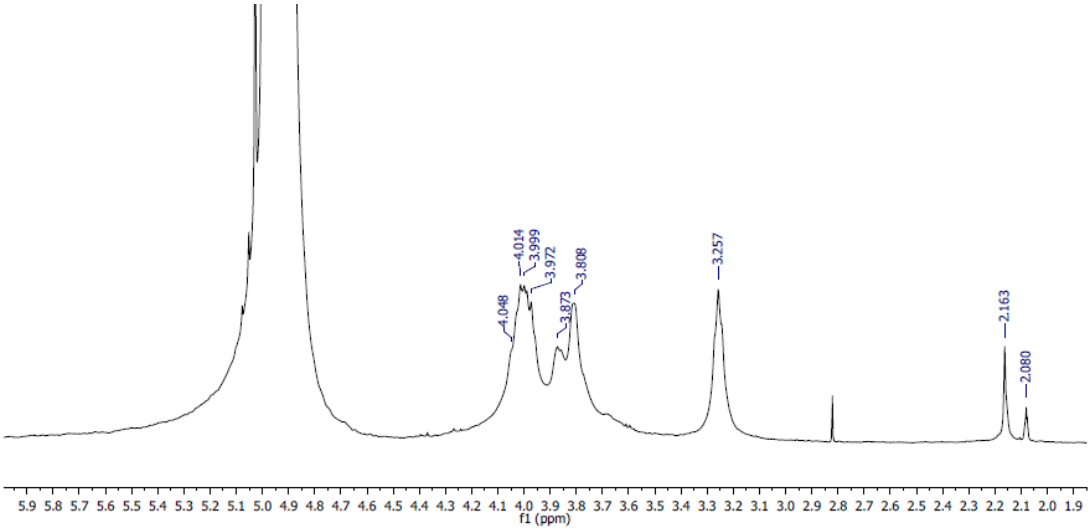

Figure 6. ^{1}H-NMR spectra of extracted chitosan

The chemical shift values of the different protons are given in Table 2. The chemical shift values of the residual protons of the solvents are as follows: [D20 (d = 4.7 ppm) and CD3COOD (d = 2.05 and 8.5 ppm)]. D2O enters into rapid exchange reactions with protons and may eliminate resonance signals such as -OH, -NH2, and NH-CO-CH3 [16].

Table 2. Chemical shifts for proton in 1% CD_3COOD/D_2O solution at 303 K

Type of proton	Position (δ, ppm)	
	Standard	*Extracted*
H_1 (H_1 of GluNAc or H-1 (A))	4.62-4.85	4.7-4.85
H_1 (H_1 of $GluNH_2$ or H-1 (D))	4.85-4.97	4.85-4.97
H_2 (H_2 of $GluNH_2$ or H-2 (D))	3.18-3.24	3,257
H_2 (H_2 of GluNAc or H-2 (A))	3.38-3.65	-
H_3 (H_3 of $GluNH_2$ or H-3 (D))	3.52-3.87	3.808-3.873
H_3 (H_3 of GluNAc or H-3 (A))	3.52-3.65	-
H_3, H_4, H_5, H_6, H_6`	3.74-4.34	3.808-4.048
$H_{N\text{-}COCH3}$	1.95-2.09	2,08
CH_3COOH (AcOH)	2.09-2.11 [15]	2,163

It can be seen from the above table that the signals of the protons in the extracted chitosan are almost identical to the data given in the literature.

3.4. ^{13}C NMR Analysis

The 13C NMR spectrum of chitosan is shown in Figure 6, which contains 8 main signals. These signals are attributed to 8 carbon atoms in N-acetyl glucosamine residues (Table-3). Each carbon signal gives a singlet signal, except C2 and C = O, a doublet signal. This is because of the influence of the quadrupole 14N nucleus of the acetamide group [17,18]. In low acetylated chitosan, a doublet signal for C4 is observed at about 86 ppm. As a result of the formation of N-acetyl groups during N-oxidation, the chemical shift values of carbon atoms change at 13C NMR spectroscopy. The C1 atoms of chitin and chitosan signal at about 100 ppm, while other carbon atoms resonate between 60 and 80 ppm. The carbon atom of the -CH2OH (C-6) group is visible at around 60 ppm. When the chitin/chitosan sample is dissolved in D2O, anomeric carbon and methyl carbon atoms are observed between 95 and 100 ppm and 15-25 ppm [16].

Table 3. Chemical shifts for carbon in 1% CD_3COOD/D_2O solution at 303 K

Type of proton	Position (δ, ppm)	
	Standard	*Extracted*
C_1	102.7-105.7	97,63
C_2	55.2-57.6	55,739
C_3	73.1-75.7	74,707
C_4	80.9-85.7	-
C_5	73.1-75.7	76,262
C_6	59.6-60.8	59,846
N-CH_3 (C_7)	22.8-23.3	21,03
N-C=O (C_8)	173.6-173.8 [15]	178,92

From the results given in the table above, we can see that the carbon signals in the 13C NMR spectrum of extracted chitosan based on *Apis mellifera* are almost identical to the data given in the literature [16,17,18].

4. Conclusions

- The structures of chitin and extracted chitosan-based on *Apis mellifera* were analyzed with FTIR spectroscopy, and it was found that the results were almost identical to the data presented in the literature;

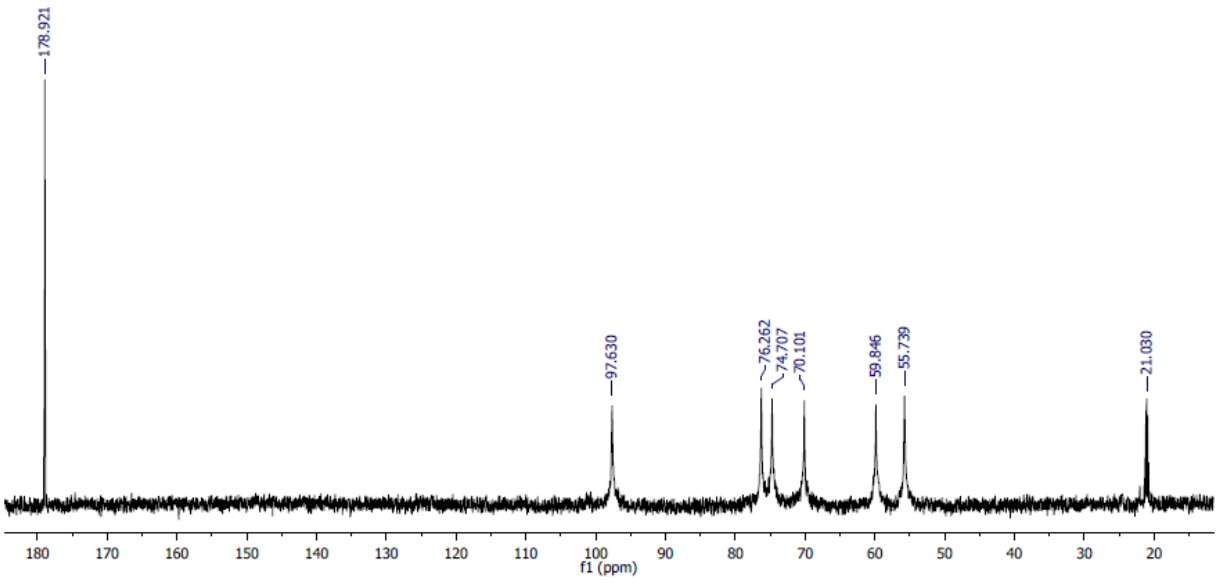

Figure 7. ^{13}C-NMR spectra of extracted chitosan

- X-ray diffraction method of the crystal structure of chitosan obtained revealed that the crystalline lattice has a tetragonal structure and a crystalline index of 31.65%. From this, we can conclude that the chitosan extracted during our research has an amorphous structure.
- The structure of the extracted chitosan was analyzed using 1H and 13C NMR spectroscopy and found that the protons and carbon-related signals in chitosan were similar to the data in the literature.

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Conflict of Interest: The authors report no conflicts of interest.

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