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Synthesis and characterization of carboxymethyl shrimp chitosan (CMSCh) from waste shrimp shell

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ABSTRACT

Chitin, the main component of shellfish such as crab and shrimp, is one of the most used biopolymers after cellulose. Today, although recycling of waste is becoming increasingly important, large quantities of seafood shells such as crab and shrimp are being destroyed around the world without much evaluation. Chitosan, which is non-toxic, biodegradable-biocompatible and has many application advantages compared to chitin, is used in many sectors, especially cosmetics, pharmaceuticals, and agriculture, as it shows superior properties compared to other biopolymers in terms of chemical and physical properties. In this study, in order to evaluate waste shrimp shells (WSS), shrimp shells were first removed from their minerals and proteins by deproteinization, demineralization, and deacetylation processes. Then, chitosan and carboxymethyl shrimp chitosan (CMSCh) were synthesized by isolation of chitin. The structures of Chitin-chitosan and CMSCh were characterized by spectroscopic methods (FT-IR, XRD, and NMR) and the deacetylation degrees of them were calculated. Also, surface morphologies and thermal properties were analyzed by SEM and DTA-TG, respectively.

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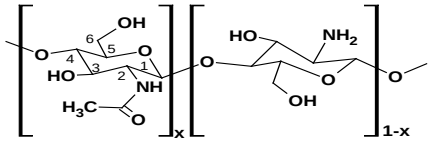
1. Introduction

Chitin, the second most common biopolymer after cellulose occurring in nature [1], is a mucopolysaccharide found in the shells of marine animals such as crab and shrimp. It is also found in the skeleton of insects and in the structure of the cell walls of fungi, mollusks, arthropods, bacteria, and some plants. The structure of chitin is similar to cellulose but contains monomer units of 2-acetamido-2-deoxy-β-D-glucose (N-acetylglucosamine) linked together by β (1 → 4) linkages. There are two hydroxyl groups per repeating monomer in the chitin and reactions take place via the more reactive primary hydroxyl group (C6-OH) because it is sterically less inhibited. In general, the shells part of sea animals such as crabs, lobsters, and shrimps consists of 30-40 % protein, 30-50 % calcium carbonate and calcium phosphate, and 20-30% chitin. The protein, calcium carbonate, and calcium phosphate in the shell of these marine animals are limited by some of the applications of chitin. Therefore the chitin is deproteinized and demineralized to remove its proteins and minerals (calcium carbonate, phosphate). The demineralized and deproteinized chitin has a light pink color due to the presence of astaxanthin pigment. The pigment in the crustacean shells forms complexes with chitin. When a bleached product is desired, this pigment can be eliminated during the decolorization step [2]. Finally, chitin is deacetylated and converted to chitosan. Deacetylastone is usually made at high temperatures with a highly concentrated sodium hydroxide solution to remove some or all the acetyl group from chitin. As a result of the deacetylation of the chitin (60 % and above), "chitosan" is obtained (Scheme 1). Where x (Scheme 1) is the degree of acetylation defined as the mole ratio of acetylated repeating units over total repeating units. If x> 0.5, the structure chitin (1→4)-2-acetamido-2-deoxy-β-D-glucan), if x <0.5 chitosan (1→ 4)-2-amino-2-deoxy-β-D-glucan).



Scheme 1. *Chitin and Chitosan*

Chitosan a product of the deacetylated chitin has attracted attention because of its biocompatibility, biodegradability, nontoxicity, antimicrobial activity, low immunogenicity, inexpensiveness, and accessibility [3, 4]. It is a multifunctional and eco-friendly compound which is known as an amino polysaccharide comprised of β-(1, 4)-2-acetamido-2-deoxy-D-glucopyranosyl and β-(1, 4) -2-amino-2-deoxy-d-glucopyranosyl units [5]. The most important advantage of the chitosan compared to the chitin is solubility in weak acid solutions owing to the protonation of its amino groups. However, chitosan has poor solubility in water due to its very stable crystalline structure arising from strong hydrogen bonds. This is limited its some application areas. Hence, the solubility of chitosan can be improved by depolymerization and its chemical modifications [6]. Chitosan has three reactive groups, primary and secondary hydroxyl groups and amino groups (Scheme 1). These reactive groups in chitosan can be easily chemically modified and changed the physical and mechanical properties. Compared with other water-soluble chitosan derivatives, carboxymethyl chitosan (CMCh) has been widely used because of its ease of synthesis, ampholytic character and possibilities for large applications. CMCh, one of the main derivatives of chitosan obtained by carboxymethylation, has unique properties such as significant biological properties, biocompatibility, gel-forming capacities, non-toxicity, biodegradability, low immunogenicity, antioxidant activities and solubility in a wide pH range [7]. Due to these properties, CMCh has used especially many biomedical applications like wound healing, bio-imaging, tissue engineering and drug/gene delivery [7]. Further, it is also frequently used in cosmetic production and composite materials because of the antimicrobial activity, moisture absorption-retention, antimicrobial and emulsion stabilizing properties of CMCh [6, 8-9].

In this study, to evaluate waste shrimp shells (WSS), shrimp shells were first removed from their minerals and proteins by deproteinization and demineralization processes. Then after the deacetylation of chitin, chitosan was obtained. Then, chitosan and carboxymethyl shrimp chitosan (CMSCh) were synthesized by isolation of chitin. The structures of chitin-chitosan and CMSCh were characterized by spectroscopic methods (FT-IR, XRD and NMR) and the deacetylation degrees of them were calculated. Also, surface morphologies and thermal properties were analyzed by SEM and DTA-TG, respectively.

1. Materials and methods

The waste of shrimp shells (WSS) used in this study was kindly obtained from the Kahramanmaras local fish market in Turkey. Other chemicals and solvents (NaOH, NaOCl, chloroacetic acid (MCA), glacial acetic acid, 2-propanol, acetone, methanol, ethanol) were analytical grade and used without further purification. The spectroscopic identification of compounds was performed using Scanning Electron Microscope (SEM) (Jeal/Neoscope Jcm-5000) at EHT = 20kV, The Fourier Transform Infrared (FT-IR) spectrums were taken from 4000 to 400 cm-1 using a Perkin Elmer Spectrum 400 Infrared Spectrophotometer with ATR apparatus, X-Ray Diffraction (XRD) patterns were analyzed using XRD diffractometer (Philips X'Pert PRO) with CuKα radiation operating, the voltage of 40 kV and current of 30 mA at monochromatic radiation (λ=154060 nm), The Thermal behaviors were measured using a TG-DTA (Seiko II, Japan) and Nuclear Magnetic Resonance (1H NMR) spectrums were recorded at 30 oC in D2O using a Bruker–200 MHz Varian spectrometer (90o pulse and 16 scans).

1.1. Isolation of chitin and chitosan

In this study, pink shrimp (Solenocera melantho) shells were collected at a local fish market to obtain chitin and chitosan. Firstly, to remove organic compounds found on the surface of shrimp shells, they were washed with tap water and then distilled water. The WSS was laid on a clean surface (Figure 1) and dried at room temperature for 48 hours. The shells were then milled with a lab-scale hammer mill and passed through a 60-80 (0.177-0.250 mm) mesh screen to obtain the uniform size. The synthesis of chitin and chitosan was carried out according to the study by Chang and Tsai [10]. Isolation of chitin involves four traditional steps: demineralization, deproteinization, decolorization and deacetylation with slight modifications. In fact, the isolation of chitin specifically consists of only two steps: demineralization and deproteinization, which involves the dissolution of calcium carbonate with HCl and the removal of proteins with NaOH, respectively

Figure 1. *Washed (A) and dryed (B) WSS*

Step 1: **Deproteinization**
In order to remove the proteins from the powder of waste shrimp shells, 1 g WSS was weighed and added to 12.5 mL 2.5 M NaOH solution and mixed at 75 ºC for 6 hours. This solution, which was brought to room temperature, was filtered with filter paper. The separated residue was washed with distilled water to neutral pH and dried.

Step 2: Demineralisation
1 g of WSS powder was added to 9 mL 1.7 M HCl acid solution and mixed at 65 ºC for 6 hours to remove minerals (calcium carbonate, phosphate). The reaction mixture was

brought to room temperature, filtered, and washed with distilled water until the pH of the residue was neutral.

Step 3: Decoloration
The decoloration step was carried out to remove pigments. The chitin powder was extracted with acetone and dried at room temperature for 2 hours. It was then bleached with 0.315% (v/v) sodium hypochlorite (NaClO) solution (containing 5.25% available chlorine), solid:solvent ratio 1:10, w/v [11].

Step 4: Deacetylation
For the N-deacetylation of the chitin obtained from decolorized chitin powder, 26 mL of 60% NaOH solution was added per gram of chitin and stirred at 107 °C for 6 hours. After the mixing process, the mixture, which lowered to room temperature, was filtered through a filter paper and washed with distilled water to neutralize the pH. The residue remaining on the filter paper was dried in an oven at 50 °C.

1.2. Determination of chitosan's deacetylation degree (DD)

The degree of deacetylation of chitosan was determined by conductometric titration. To determine the DD, 5 g of chitosan was kept in the desiccator for 3 hours to bring it to a constant weight. 4 g chitosan (weight on a dry basis) was dissolved with 20 mL of 0.1 M HCI acid and the solution was transferred 250 ml flask for titration. Then, 2 drops of methyl orange were added to the solution, and the solution was mixed until the color turned pink. This solution, which was stirred until its color turned pink to orange, was titrated with 0.1 M NaOH. DD value was calculated according to the formula below by recording the amount of NaOH consumed in the titration [12-13].

$$DD(\%) = 2{,}03x\frac{V2 - V1}{m + 0{,}0042(V2 - V1)}$$

Where;
m= weight of sample (g)
V1= volume of NaOH added
V2= volume of HCI added
2.03= coefficient resulting from the molecular weight of chitin monomer unit
0.0042= coefficient resulting from the difference between molecular weights of chitin and chitosan monomer units.

2.3. Preparation of carboxymethyl shrimp chitosan (CMSCh)

CMSCh was synthesized by treating chitosan with aqueous sodium hydroxide solution and then reacting with monochloroacetic acid (MCA) according to the study of Kusuma et al. [14]. 3 grams of chitosan was suspended in 65 mL of isopropyl alcohol and stirred at room temperature for 10 minutes. 22 mL of 40% NaOH solution (w/w) was added to this suspension and stirred for another 30 minutes. Then 15 grams of MCA was added to this basic suspension and stirred at 45 °C for 3 hours. The solid residue obtained by filtration of this cooled solution was washed with pure methanol and neutralized with glacial acetic acid. It was then washed with 80% methanol and several times absolute ethanol and dried to constant weight. The images of the obtained chitin, chitosan and CMSCh, and their formulas are given in Figure 2 and in Figure 3, respectively.

Figure 2. *Imagines of dried shrimp shell, chitin, chitosan and carboxymethyl shrimp chitosan*

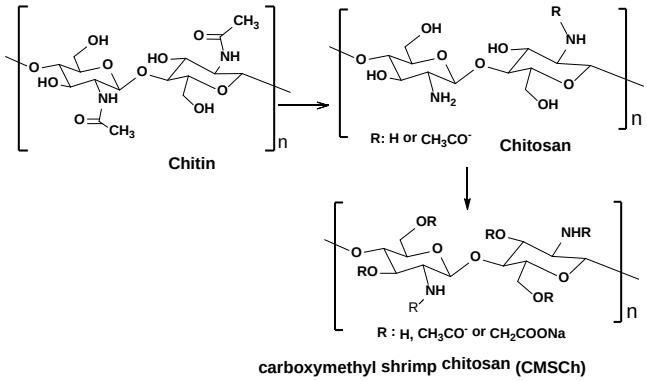


Figure 3. *Chitin, Chitosan and CMSCh*

2. Result and discussion

Although chitin, which is the second most abundant in nature after cellulose as a natural polymer, is widely used in many areas, some problems may be encountered in practice due to its tight supra-molecular structure. For this reason, it is preferred to use chitosan, which is obtained as a result of deacetylation of chitin, instead of chitin. Some parameters such as deacetylation degree and molecular weight, especially pH, viscosity and color are the properties that determine the use of chitosan. In our study, shrimp shells were firstly purified from minerals and proteins by deproteinization and demineralization processes. Demineralization consists in the removal of minerals, primarily calcium carbonate. Its generally performed by acid treatment using HCl, HNO_3, H_2SO_4, CH_3COOH and HCOOH [15, 16]. Conventionally, demineralization is accomplished using dilute hydrochloric acid at different concentrations (up to 10% w/v) at room temperature, during different incubations time. Demineralization is easily achieved because it involves the decomposition of calcium carbonate into the water-soluble calcium salts with the release of carbon dioxide as shown in the following equation [17]:

$$2HCl + CaCO_3 \rightarrow CaCl_2 + H_2O + CO_2\uparrow$$

Most of the other minerals present in the shellfish cuticle react similarly and give soluble salts in presence of acid. Then, salts can be easily separated by filtration of the chitin solid phase followed by washing using deionized water. Another step is the decolorization of chitin to remove decolorizing agents. The color of chitin products is varied from cream white to intermediate pink color. Because chitin has a light pink or near-white color due to the presence of astaxanthin pigment. For commercial acceptability, the chitin produced from crustacean sources needs to be decolorized which is a process to remove astaxanthins and pigments or bleached to yield cream white chitin powder [15]. When a bleached product is desired, this pigment can be eliminated during the decolorization step [2, 11]. In the last step, chitosan was obtained from the chitin by deacetylation process. Then, obtained chitosan CMSCh was synthesized from obtained chitosan thus waste shrimp shells were evaluated by the synthesis of carboxymethyl shrimp chitosan (CMSCh) from chitin-chitosan. The structures of chitin-chitosan and CMSCh were characterized by spectroscopic methods such as FT-IR, XRD, and NMR, and deacetylation degrees were calculated. In addition, surface morphologies and thermal properties were investigated by SEM and DTA-TG, respectively.

2.1. Degree of deacetylation

The physiological properties of chitosan, especially solubility and molecular weight are determined by its degree of deacetylation [1]. Deacetylation is the process to convert chitin to chitosan by the removal of acetyl groups from the molecular chain of chitin. It is generally obtained by treatment with concentrated sodium or potassium hydroxide solution (40–50 %) at 100 ºC or higher, for 30 min or longer to remove some or all of the acetyl groups from the polymer. Thus, the chitosan compound having a highly chemically reactive amino group (-NH2) is formed. The process of deacetylation is carried out to different degrees depending on its applications. The N-acetyl groups cannot be removed by acidic reaction without hydrolysis of the polysaccharide, thus, alkaline methods must be employed for N-deacetylation [18]. Different researches have reported that the degree of solubility of chitosan is higher for higher degrees of deacetylation (DD) and lower molecular weight [19]. The degree of deacetylation of chitosan ranges from 56 to 99 % with an average of 80 % depending on the crustacean and the preparation methods [20]. In this study, when the acetylation time was 3 hours (chitosan-4), the highest acetylation degree (73.88%) was obtained. As the reaction time was longer, the degree of deacetylation started to decrease (Table 1).

Table 1. *Degrees of deacetylation depending on the reaction time of the obtained chitosan*

Samples	DD (%)
chitosan -1 (treated for 0.5 h)	57.80
chitosan -2 (treated for 1 h)	65.57
chitosan -3 (treated for 2 h)	73.45
chitosan -4 (treated for 3 h)	73.88
chitosan -5 (treated for 4 h)	72.21
chitosan -6 (treated for 5 h)	65.45

Our results proved that CMSCh prepared at temperatures of 45 oC were soluble in water as in other literature [21-22]. Compared with chitosan, the solubility of CMCh in an aqueous solution could be explained with the introduction of the carboxymethyl group. The properties of chitosan and carboxymethyl shrimp chitosan are compared in Table 2.

Table 2. *Comparative characteristic of chitosan and CMSCh*

Characteristic	Chitosan	CMSCh-4
Appearance	powder	powder
Colour	pale white	pure white
Odor	light	odorless
DD, %	81	-
Solubility	in acidic medium	in water

2.2. Scanning electron microscopy (SEM) of Chitosan and CMSCh

SEM analysis results for chitosan and CMSCh-4 were shown in Figure 4. Based on this SEM analysis, while the chitosan surface was smooth and flat and had no fibrous structure CMCh surface was lamellar and microstructured. The fact that CMSCh-4 has an uneven surface property indicates the presence of a new formation adhering to the chitosan surface [14, 23]. Increasing magnification from 1000× to 3000× was revealed the recessed and porous surface of CMCh-4.

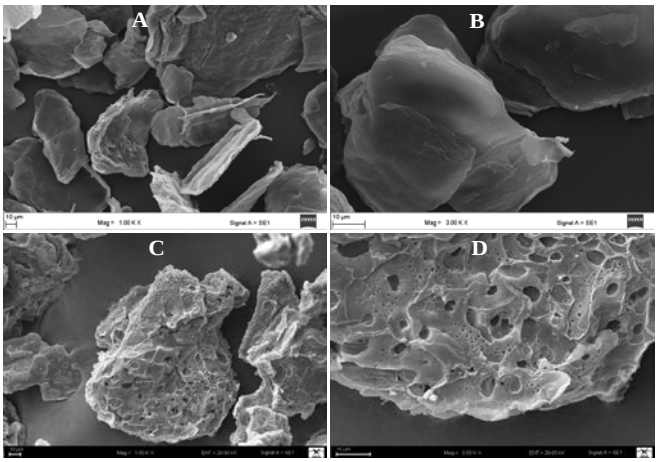


Figure 4. *SEM images of chitosan with a magnification of (A) 1000 and (B) 3000 times and CMSCh-4; (C) 1000 and (D) 3000 times*

2.3. FTIR spectrums of Chitosan and CMSCh

FTIR spectra of chitosan and CMSCh-4 were shown in Figure 5. The FT-IR spectrum of chitosan showed a wide and broad absorption band between 3401 and 3250 cm^{-1} due to -OH and -NH_2 vibration [24]. This vibration band can be attributed to the presence of free amine formed in chitosan as a result of deacetylation. The significant peaks at around 2971 cm^{-1} are assigned to the asymmetrical and symmetrical stretching vibrations of methylene groups confirm the insertion of long aliphatic chains in the CMSCh molecular structure [25]. A band at 1590 cm^{-1} was observed, which is attributed to the angular deformation of the N–H bonds of the amino group. The band at 1373 cm^{-1} due to the symmetrical angular deformation of CH_3, and the appearance of peaks at 1322 cm^{-1} correspondence to the carboxyl groups overlap with the deforming vibration of NH_2, C–O-C and C-OH [26-27]. The band corresponding to the polysaccharide skeleton including vibrations of the glycoside bonds, C–O and C–O–C asymmetric bridge-O-stretch at 1148 cm^{-1}. The peak at 1056 cm^{-1} was attributed C–O bend stretched and the second hydroxyl groups of a bridge –O– stretch was appeared at 1056 cm^{-1} [25]. The spectra of CMSCh-4 were similar to that of the chitosan (Figure 5). The occurrence of an intense band at 1589 cm^{-1} and a moderate band at 1409 cm^{-1} due to the symmetric and asymmetric axial deformations of COO^-, respectively, confirms the introduction of the carboxymethyl groups [24]. The appearance of a peak at 1409 cm^{-1} corresponding to the carboxyl group –CH- and CH_3 groups are indicate the carboxymethylation on both the amino and hydroxyl groups of chitosan [27-30]. These results showed that the carboxymethylation was achieved successfully.

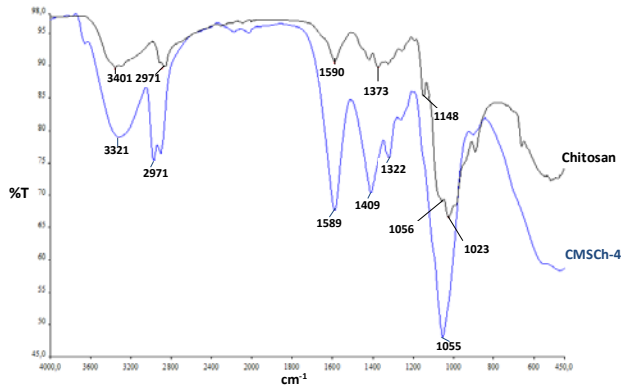


Figure 5. *FTIR spectra of chitosan and CMSCh-4*

2.4. ^{1}H- NMR spectrum of CMCh

The ^{1}H-NMR spectrum of prepared CMSCh-4 was illustrated in Figure 6. In the ^{1}H-NMR spectrum of CMSCh, signal at 3.8-3.2 ppm was observed; the signal was evidence of N-carboxymethylation. The signal observed between 1.80 and 1.90 ppm corresponds to the hydrogen bonded to the C2 glucosamine ring, while the signals between 3.25 and 3.83 ppm probably as a consequence of the carboxymethylation of the hydroxyl groups bonded to the carbon atoms C3, C4, C5 or C6 of the glucopyranose that are overlapped, and the signal assigned at 4.8 ppm was due to the solvent [24, 26]. The signal centered at 1.80 ppm corresponds to the hydrogens of the methyl moieties belonging to the acetamido groups. Importantly signal that was observed at 8.36 ppm is assigned to the carbonyl carbons of carboxymethyl groups [31], while the one detected at 8.34 ppm corresponds to the carbonyl carbon of -$COCH_3$ of the parent chitosan.

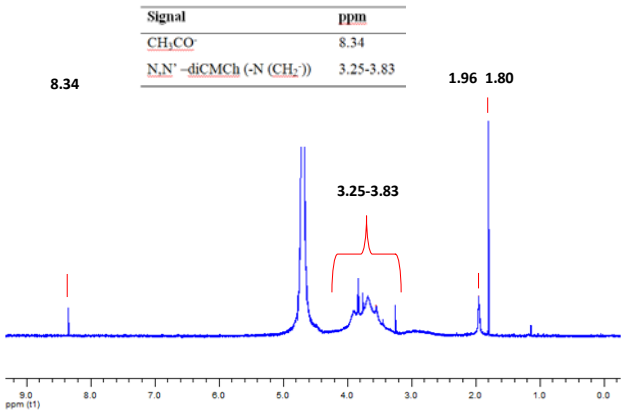


Figure 6. *^{1}H NMR of CMSCh-4*

2.5. XRD spectrums of Chitosan and CMSCh

XRD patterns of chitosan and CMSCh-4 were shown in Figure 7. The characteristic peaks of chitosan appeared a range of 2θ=20° while the characteristic peaks of CMSCh appeared a range of 32º. In the chitosan, the characteristic peak seen at 20° was decreased significantly after carboxymethylation [23]. Based on the obtained results of the XRD diffractogram, it can be said that the sharp peaks that exist on chitosan appear at different angles when compared to the sharp peaks found in CMSCh-4. CMSCh was recognized in some literature by the appearance of characteristic peaks that are less sharp than the characteristic peaks of chitosan at about 32º [14, 32]. These results show that the addition of a large number of substituents to the chitosan polymer, which disrupts the hydrogen bond and constitutes a significant steric barrier in chitosan, significantly changes the crystallinity pattern. The presence of carboxymethyl groups which substitute the hydrogen atoms of the hydroxyl and amino groups of chitosan, decrease the formation of hydrogen bonds. This is due to the less number of hydrogen bonds formed between carboxymethyl shrimp chitosan molecules than chitosan.

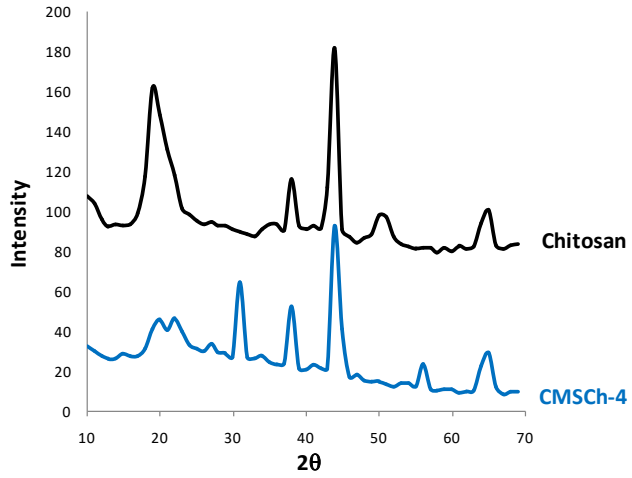


Figure 7. *XRD pattern of Chitosan and CMSCh-4*

2.6. TG-DTA spectrums of Chitosan and CMSCh

The thermal behaviors of chitosan and CMSCh were investigated at a temperature range of 30-600º C. As can be seen in Figure 8, the thermal decomposition progress for the initial weight loss in chitosan 8% and CMSCh-4 7% was observed at 100 °C, as a consequence of water evaporation [29]. The high decomposition in both was around 200-350 ºC. In the second stage, the weight loss of chitosan and CMSCh-4 is related to the decomposition of non-volatile components [33-34]. Endothermic peaks occurred at 291 °C in chitosan, 287 ºC in CMCh-4. Moreover, the mass loss is 31.12% and 19.44%, respectively. Chitosan was appeared to lose more weight than CMSCh-4 when the temperature rises above 350 ºC. This can be attributed to the degradation of the part of the molecule that is deacetylated [29]. It was observed that the weight loss of chitosan at the final temperature (590 ºC) was 99% while the CMSCh-4 was 50%. After TGA/DTA analysis can be concluded that CMSCh is suitable to be used in heating. All these observations revealed the higher thermal stability of synthesized CMSCh for potential application in some areas such as food processing and pharmaceutical preparations [27].

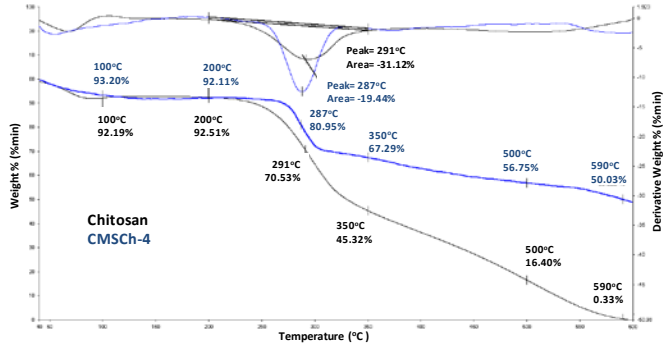


Figure 8. *TG-DTA of Chitosan and CMSCh-4*

3. Conclusions

Today, although recycling of waste is becoming increasingly important, large quantities of seafood shells such as crab and shrimp are being destroyed around the world without much evaluation. Chitosan, which is non-toxic, biodegradable-biocompatible and has many application advantages compared to chitin, is used in many sectors, especially cosmetics, pharmaceuticals and agriculture, as it shows superior properties compared to other biopolymers in terms of chemical and physical properties. For this purpose, Recycling of shrimp shells was evaluated by synthesizing carboxymethyl shrimp chitosan. Optimum conditions in the isolation of chitin-chitosan and synthesis of CMSCh have been compared with the literature. When the acetylation time was 3 hours (chitosan-4), the highest acetylation degree (73.88%) was obtained. It is understood that these natural polymers, whose structure is characterized by spectroscopic methods, can be used in many areas mentioned above.

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