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Persian gulf β-chitin extraction from *sepia pharaonis sp.* cuttlebone and preparation of its derivatives

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ABSTRACT

Persian Gulf β-chitin was extracted from the cuttlebone of Sepia Pharaonis and chitosan was prepared through deacetylation using microwave technique. β- Chitin and β-chitosan were characterized for their structural and physical (CHN, DDA, FT-IR, NMR, XRD and Viscometric analysis) properties. The purity, DDA and molecular weight of present chitosan were found 85.3%, 90% and 350.06 kDa. Further, acyl chitosan derivatives were prepared by the reaction of β-chitosan with several derivatives of acyl chlorides.

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1. Introduction

Chitin is one of the most abundant polysaccharide in nature, after cellulose. As shown in Fig. 1, chitin is a linear biopolymer composed by 2-acetamide-2-deoxi-D-glucopyranose units linked by β(1→4) glycosidic bonds, whose chains interact by numerous hydrogen bonds involving its hydroxyl, amine and carbonyl groups leading to the formation of micro fibrils (Lehane, 1997).

Three different polymorphs of chitin are found in nature, the α-chitin being the most common structure and corresponding to a tightly compacted orthorhombic cell formed by alternated sheets of parallel and antiparallel chains (Minke and Blackwell, 1978). The β-chitin adopts a monoclinic unity cell where the polysaccharide chains are disposed in parallel fashion (Gardner and Blackwell, 1975) and although the structure of γ-chitin has not been completely identified, an arrangement of two parallel and one antiparallel sheet has been proposed (Rudall, 1963).

Chitin is mainly used for production of chitosan by a deacetylation reaction usually obtained in alkaline medium. Chitin is usually isolated from the exoskeletons of crustaceans, shrimps and crabs in α form (Austin, Castle, & Albisetti, 1989; Tajik, Moradi, Razavi Rohani, Erfani & Shokouhi Sabet Jalali, 2008; Musarrat, Williams, & Tverezovskay, 2013). Squid and cuttlebone are another important sources of chitin in which it exists in the β form which was found to be more amenable for deacetylation. It also shows higher solubility, higher reactivity and higher affinity towards solvents and swelling than α-chitin due to much weaker intermolecular hydrogen bonding ascribable to the parallel arrangement of the main chains (Hunt and Elsherief, 1990). Furthermore Cuttlebone is a highly porous hard tissue in cuttlefish, which functions as a rigid buoyant tank in the animal. Its framework is an inorganic–organic composite composed of aragonite, protein and β-chitin (Birchall and Thomas, 1983). The application of chitin and chitosan include cosmetics, agriculture, food, biomedical, wound dressings, drug delivery, and textile, as chelating agents and refinement industrial effluents (Henriksen, Sminstad, & Karslen, 1994; Imai, Shiraishi, Saito, & Otagiri, 1991; Chassarya, Vincenta, Marcanob, Macaskiec, & Guibala, 2005). Due to the properties of β-chitin, the studies on its extraction and characterization from squid and cuttlefish bones have been more frequent in the last years (Kurita et al., 1993; Entsar, Khaled, & Maher, 2008; Al Sagheer, Al-Sughayer, Muslim, & Elsabee, 2009; Weaver et al., 2011; Vino, Ramasamy, Shanmugam, & Shanmugam, 2012; Subhapradha et al., 2013; Ramasamy, Subhapradha, Shanmugam, & Shanmugam, 2014).

Several techniques to extract chitin from different sources have been reported. The most common method is referred to as the chemical procedure. The chemical method for isolation of chitin from the waste involves various major steps: extraction of protein matter in alkaline medium (deproteinization) and it is traditionally

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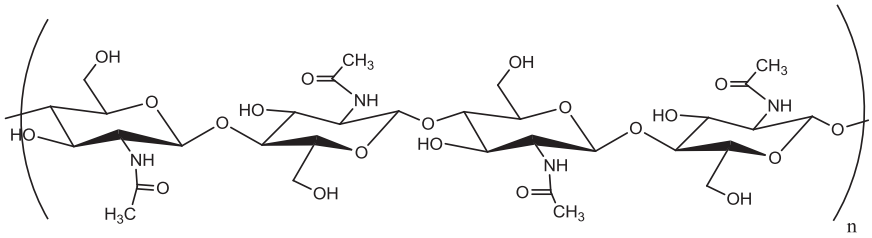

Fig. 1. Structure of β-chitin.

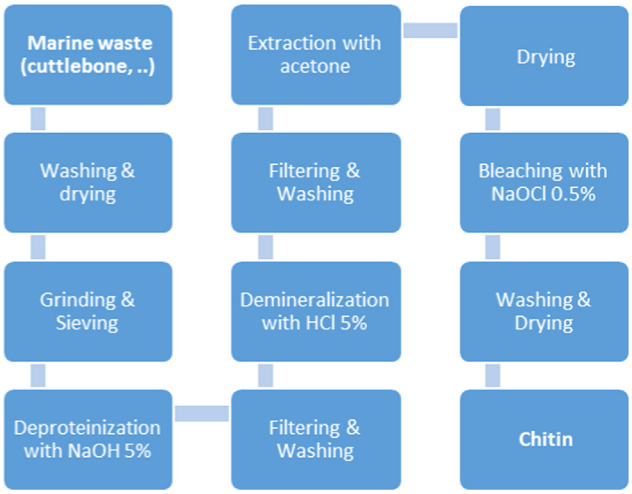

Fig. 2. Overall process for preparation of chitin from cuttlebone.

done by treating the waste with aqueous solutions of NaOH. Elimination of inorganic matter ($CaCO_3$) in dilute acidic medium (demineralization), which is accomplished by using HCl and finally bleaching in dilute NaOCl afforded pure chitin as shown in Fig. 2 (Ogasawara, Shenton, Davis, & Mann, 2000).

Finally Chitin is converted into more applicable chitosan, a structural modification of chitin often performed by deacetylation, using alkaline hydrolysis. It is soluble in aqueous acidic medium due to the presence of amino groups (Illanes et al., 1990) (Fig. 3).

In continuation of our studies on the marine natural compounds (Shushizadeh, Mostoufi, & Fakhrian, 2012), it was decided to report the isolation of chitin from the cuttlebone of *Sepia Pharaonis sp.* in Persian Gulf-Bushehr of Iran, deacetylation of it for the formation of more useful chitosan and finally the study of some acyl-chitosan preparation.

2. Materials and methods

2.1. Reagents and materials

All starting materials were purchased from Merck and Aldrich Companies. The IR spectra were recorded on a Perkin-Elmer RXI infrared spectrometer. The XRD was recorded on X-Ray Diffraction ID3003 SIEFRET Made in Germany. The CHN analyzer was recorded on CHNSO ECS4010 elemental combustion made in Italy. TLC accomplished the purity of substrates and reactions monitored on silica gel (Merck, Germany) Polygram SIGL/UV254 plates. The melting points are uncorrected.

2.2. Preparation of cuttlebone (sepia pharaonis sp.)

In this study cuttlebone (*S. Pharaonis sp.*) collected from Bushehr, Iran (North coast of Persian Gulf), in October 2012, and were washed several times using deionized water to remove some extraneous and salts. Then, they were dried in an oven at 60 °C for 48 h. The dried cuttlebone was ground and the particles were separated according to their sieves. Identification of cuttlefish bone was carried out kindly by Khoramshahr marine science and Technology University. It was indicated as *S. Pharaonis* sp. as shown in Fig. 4.

2.3. Extraction of chitin and conversion into chitosan

Chitin was extracted from the cuttlebone of *S. Pharaonis sp.* by demineralization, deproteinization and decolorization following the method described by Takiguchi (1991). For this reason, One hundred grams of cuttlebone powder was immersed in 1000 ml of 10% (*w*/*w*) HCl at room temperature (25 °C) for 24 h. After filtration with filter paper, the residue was washed with distilled water to neutral. Then the residue was immersed in 1000 ml of 10% (*w*/*w*) NaOH at 60 °C for 24 h for deproteination. The proteins were removed by filtration. Distilled water was used to wash the residue to neutral.

Then the cuttlebone residue was subjected to the above program for two times. 250 ml of 95% and absolute ethanol were sequentially used to remove ethanol-soluble substances from the obtained crude chitin and to dehydrate. An air oven was taken to dry the chitin (200 g) at 50 °C overnight.

Chitin, thus obtained from cuttlebone was converted into chitosan by the process of deacetylation according to the method of Sahu, Goswami, and Bora (2009) using 40% aqueous NaOH and microwave technique.

2.4. Preparation of chitosan

The chitin (10 g) was put into ceramic mortar and 100 ml of 50% NaOH at 100 °C for 120 min to prepare crude chitosan (w/v)

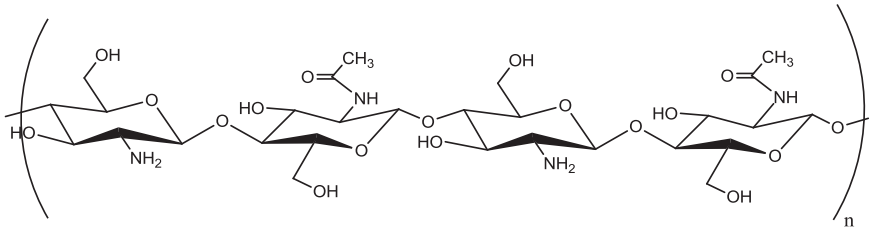

Fig. 3. Structure of β-chitosan.

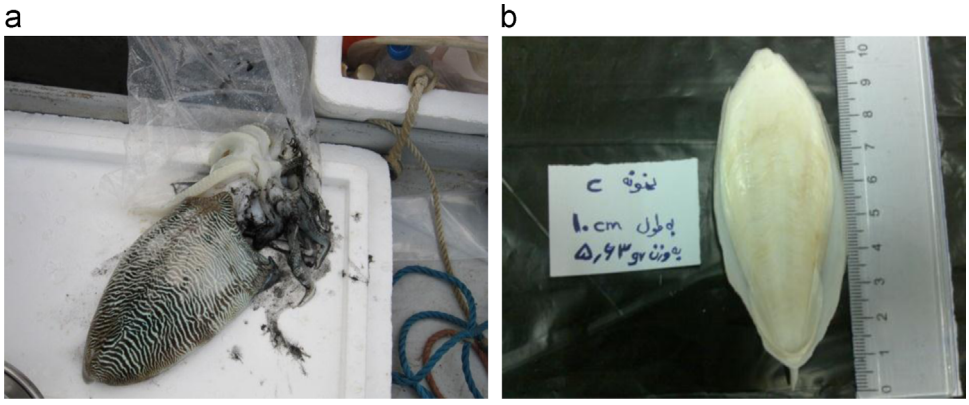

Fig. 4. (a) Cuttle fish (*Sepia Pharaonis* sp.)-left image; (b) Cuttlebone-right image.

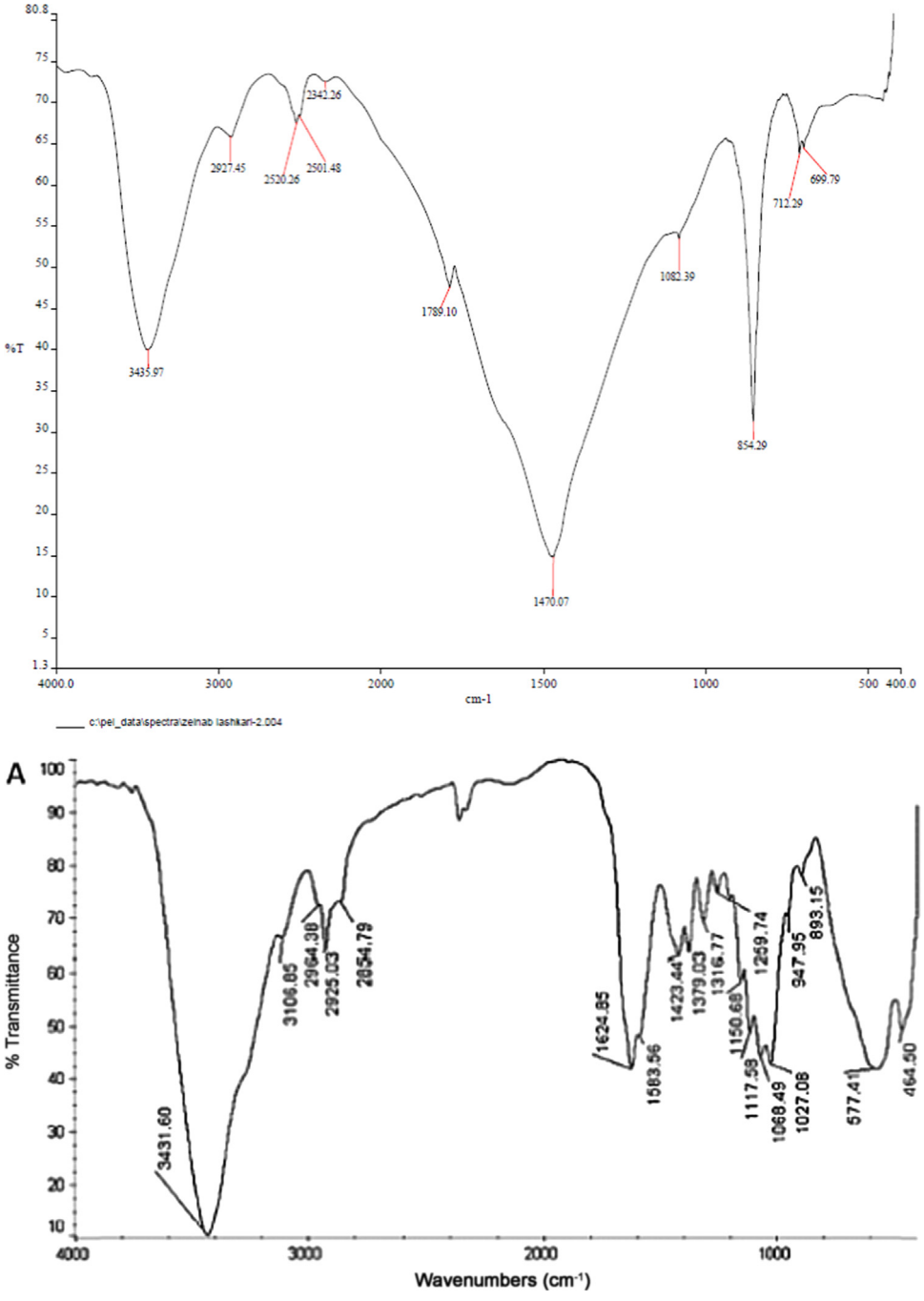

Fig. 5. FT-IR spectrum of sepia pharaonis chitin (upper)- standard chitin (down).

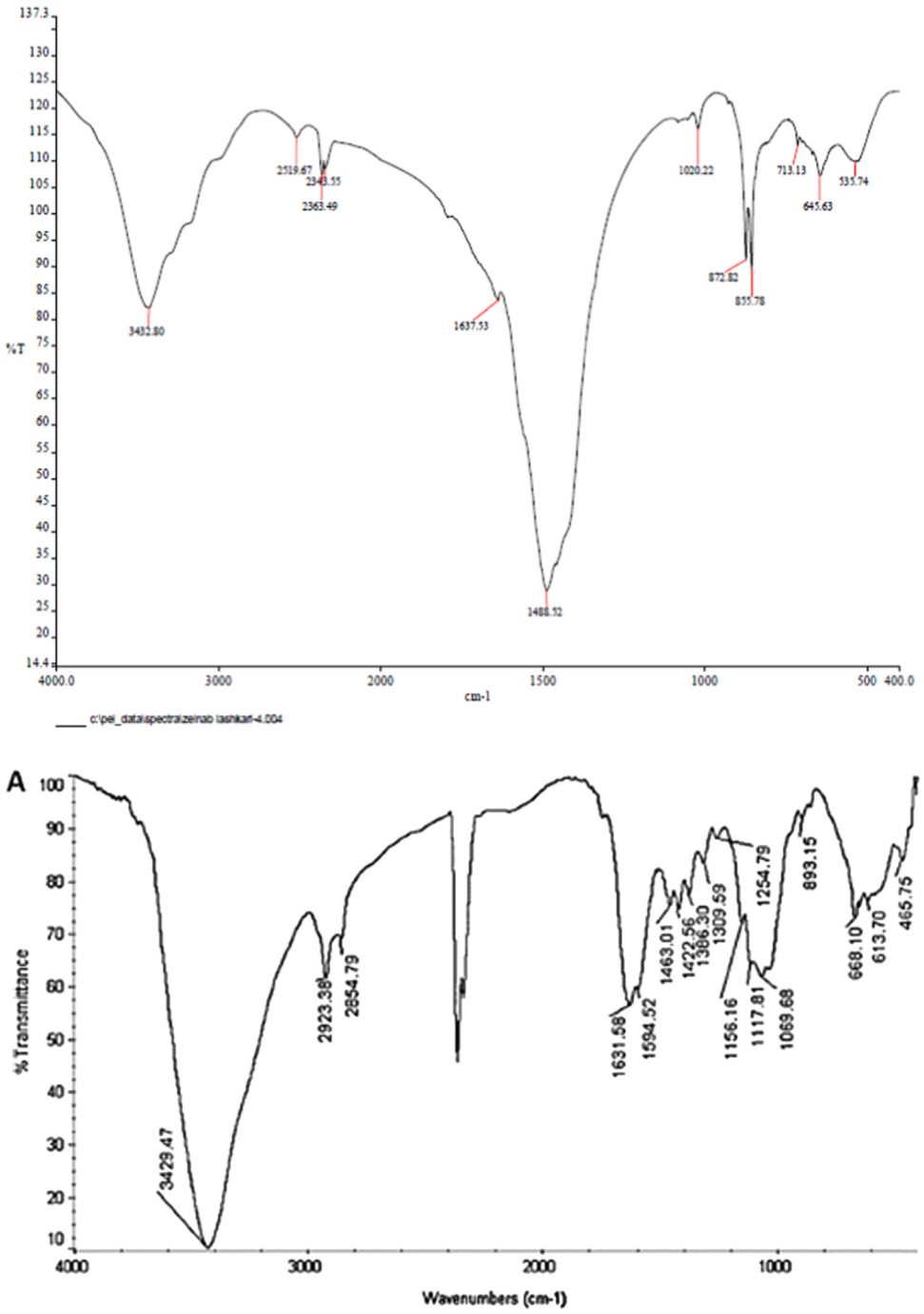

Fig. 6. FT-IR spectrum of sepia pharaonis chitosan (upper)- standard chitosan (down).

was added and mixed. The ceramic mortar was placed on the center of the turntable of the microwave oven (Milestone microwave oven for synthesis, Model MICROSYNTH, 1000 W, Made in Italy) and irradiated for 15 min at 1000 W. The mixtures were filtered and the residues were washed with distilled water until neutralization, then dried in a hot air oven at 40 °C until constant dry weight and stored until further analysis. The crude chitosan (5 g) was obtained by drying in an air oven at 50 °C overnight.

2.5. General procedure for acylation of chitosan

Chitosan powder (0.5 g) was dissolved in 0.5% (w/v) of aq HOAc (50 mL). With stirring, 2% (w/v) of aq $NaHCO_3$ (50 mL) was added drop wise over a 10-min period to obtain transparent gel. The obtained gel was washed with water till a neutral condition was obtained, then soaked in pyridine for one day, and filtered to obtain the activated chitosan. Then, the activated chitosan was added to round flask and dissolved in DMF (20 mL), and acyl chloride (5.4 g)- which is prepared from general reaction of carboxylic acid and $SOCl_2$-dissolved in pyridine (10 mL) was added drop wise to the flask in a few minutes. After stirring for half an hour, the flask was placed at reflux condition for 6 h time and 70 °C temperature. After this time, the mixture was poured into methanol (100 mL), and the precipitated product was filtered, washed with methanol and dried under vacuum for 24 h.

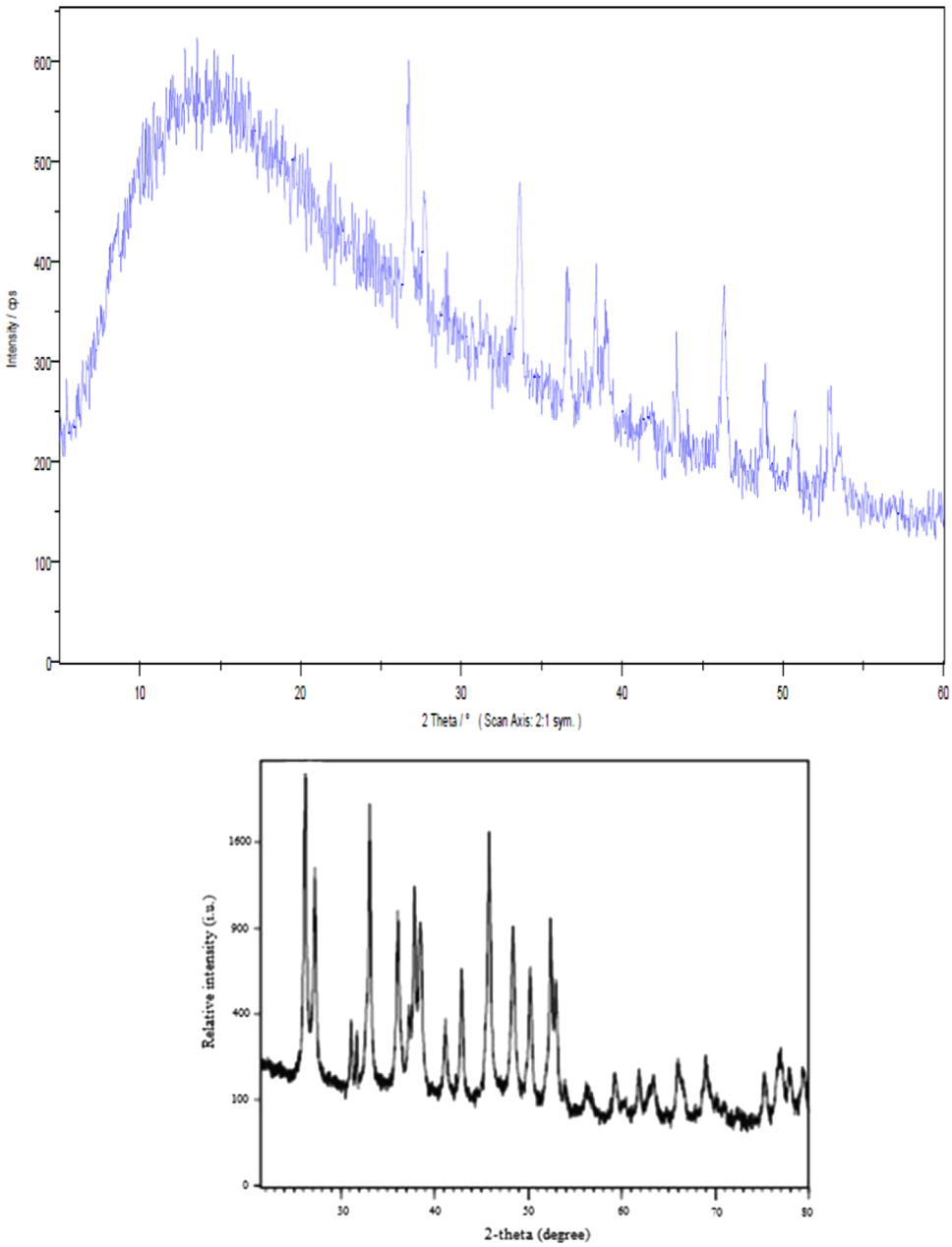

Fig. 7. The XRD spectrum of sepia pharaonis cuttlebone (upper)- reported in reference (down).

2.6. Determination of DA (degree of N-acetylation of chitosan)

The degree of N-acetylation of the samples was calculated based on Baxter method using the following equation (Baxter, Dillon, Taylor, & Roberts, 1992):

$$\%N - \text{acetylation} = \left(A_{1655}/A_{3450}\right) \times 115$$

2.7. Elemental analysis (carbon, hydrogen and nitrogen)

Elemental analysis (CHN) of sample was done using CHNSO ECS4010 elemental combustion.

2.8. Ft-IR

All samples were freeze-dried overnight, made into KBr disks, and the FTIR spectra obtained with a Perkin-Elmer RXI infrared spectrophotometer in the region between 400 and 4000 cm^{-1}.

2.9. X-ray diffraction (XRD)

The intensity of the diffracted X-rays (X-Ray Diffraction ID3003 SIEFRET) was measured as a function of the diffraction angle 2θ and the specimen's orientation. This diffraction pattern was used to identify the specimen's crystalline phases and to measure its structural properties (which are measured with great accuracy) and the size and orientation of crystallites (small crystalline regions).

2.10. Viscosity measurements (Mao et al., 2004)

For the determination of molecular weight using viscosity, the chitosan solutions were prepared in acetate buffer, pH 4.6. The efflux time of solutions was measured in triplicate using Ostwald

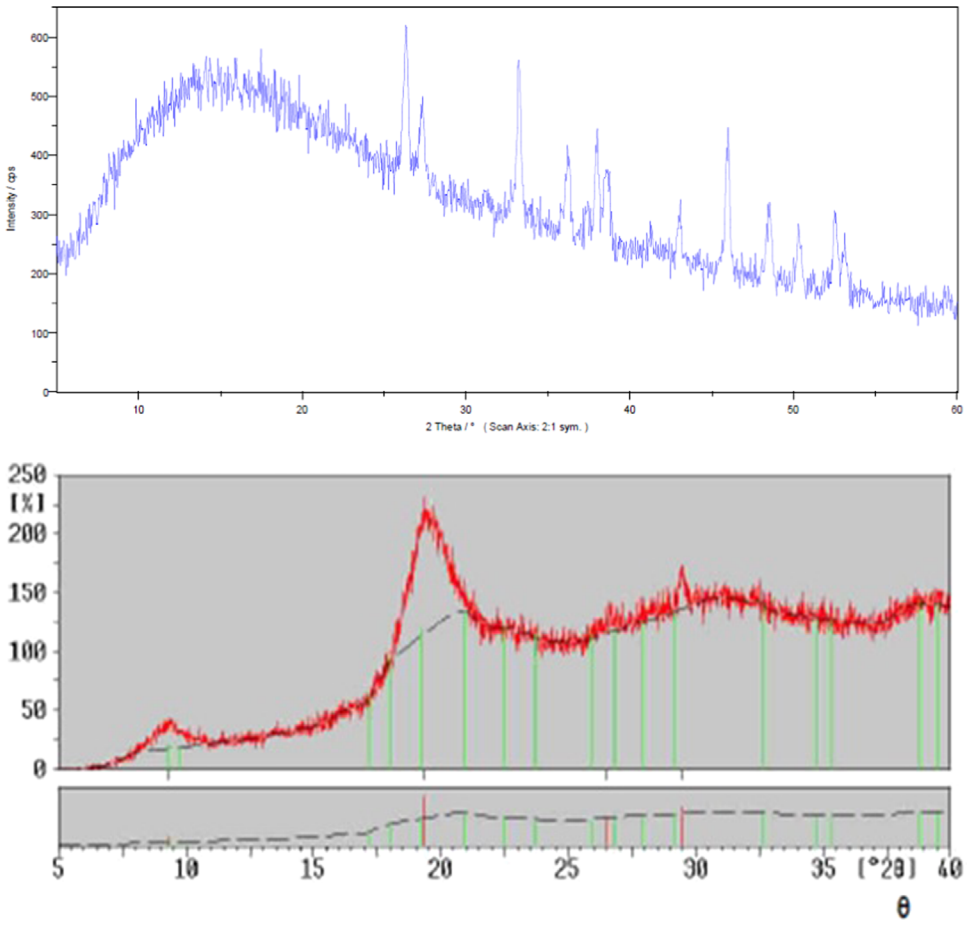

Fig. 8. The XRD spectrum of sepia pharaonis chitin (upper)- chitin reported in reference (down).

capillary viscometer at an ambient temperature. The viscosity average molecular weight of chitosan was calculated using Mark–Houwink equation:

$$[\eta] = K(M_v)^{\alpha}$$

where, $[\eta]$ is the intrinsic viscosity of the depolymerized chitosan, K and α are constants for given solute system and temperature; $K = 1.38 \times 10^{-5}$ and $\alpha = 0.85$.

3. Results and discussion

3.1. Characterization of chitin and chitosan

3.1.1. Yield of chitin and chitosan

Many researchers have reported that the yield of chitin differ between species to species, 70% (Takiguchi, 1991) in krill, 90% and 60% (Brzeski, 1987) in krill and crab, and 29.87% (Ramasamy et al., 2014) S. kobiensis cuttlebone respectively. In the present investigation the yield of chitin from the *S. pharaonis* cuttlebone of was found to be 20%. Chitin polymer is insoluble in water and hence hampers its wide use. So as to improve its solubility and wide use many derivatives of chitin have been prepared further by keeping chitosan as a base-material, is prepared by deacetylation of chitin in the presence of concentrated NaOH solution. In this order, the following are some of the important attempts made by several researchers on chitin extracted from a variety of sources. The percentage yield of chitosan from the chitin of various sources of cephalopod mollusk was found as Sepia prashadi 15% (Vino, Ramasamy, Vairamani, & Shanmugam, 2011) and S. kobiensis 43.77% (Ramasamy et al., 2014). Whereas in the present study, Sepia pharaonis cuttlebone recorded a moderate yield of 50% which is higher than the yield of chitosan from the other cuttlefish species.

3.1.2. Degree of deacetylation

Chitin samples have different degrees of acetylation (DDA) depending on their sources of origin and mode of isolation (Ramasamy et al., 2014). The degree of deacetylation of chitosan from S. pharaonis cuttlebone was calculated approximately 90% (determined through IR spectrum) in contrast with 85.55% for *Sepia Kobiensis,* represents the removal of maximum number of acetyl groups which shows the purity of chitosan.

3.1.3. Elemental analysis (C, H and N)

The carbon, hydrogen and nitrogen of chitosan obtained from Sepia kobiensis (Ramasamy et al., 2014) found as 39.30%, 3.63% and 7.70% respectively. Whereas in the present work, carbon, hydrogen and nitrogen content of chitosan was given 39.02%, 5.82% and 10.20% respectively.

After conversion of the nitrogen content to the chitosan content by a theoretical factor of 161/14, which is the molecular weight ratio of a monomer ($C_6H_{11}O_4N = 161$) to nitrogen ($N = 14$) in the chitosan (general formula $[C_6H_{11}O_4N]_n$), the chitosan contents, namely the purities were 109%, 102% and 101% for chitosans at 120 min, China and Sigma, respectively. After N-deacetylation, the product with a nitrogen content of more than 7.0% was considered as chitosan (Muzzarelli & Rocchetti, 1985). After calculation, it seems that the purity for the product to be considered as chitosan was 85.3%. Accordingly, all crab chitosans were nearly pure

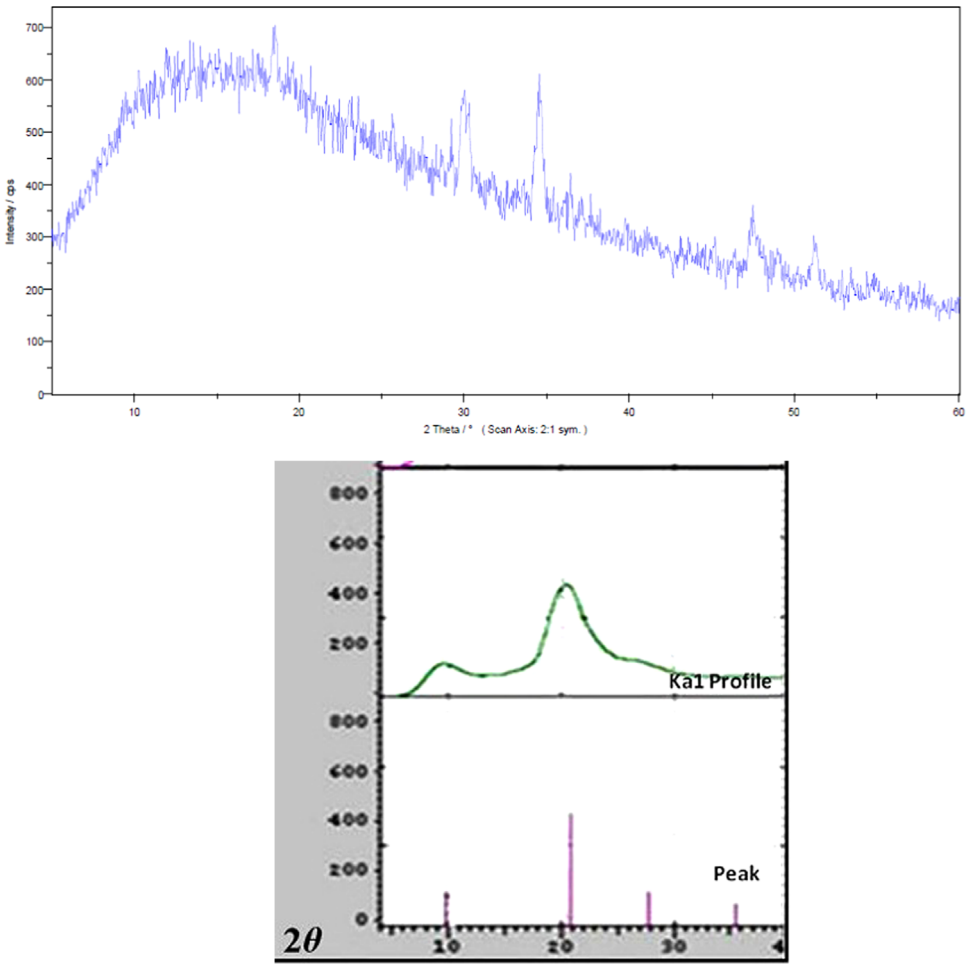

Fig. 9. The XRD spectrum of sepia pharaonis chitosan (upper)- reported in reference (down).

chitosans.

3.2. Spectral data

3.2.1. FT-IR spectral analysis of chitin and chitosan

The FT-IR spectral bands of S. pharaonis chitin observed at 3443.49 and 2927.45 cm^{-1} correspond to the vibrational stretching of OH, NH and C–H groups respectively. The wide peak at 1675.06 cm^{-1} is corresponded to C=O of amide group which is produced from the hydrogen interactions between OH and C=O. Four peaks at 1383.94, 1559.34, 1313.43 and 764.72 cm^{-1} are corresponded to structural units of chitin. In comparison the FT-IR spectrum of the standard chitin showed bands at 3431 cm^{-1}, 2925 cm^{-1}, 1583 cm^{-1} and 1423 cm^{-1} indicates H-bonded OH stretching, aliphatic CH stretching, NH bending and pyranose ring bending respectively (Subhapradha et al., 2013; Brzeski, 1987).

The FT-IR spectrum of the standard chitosan showed bands of 3429 cm^{-1}, 2923 cm^{-1}, 1631 cm^{-1}, 1309 $cm^{-1}$and 1156 cm^{-1} indicating the H-bonded NH_2 and OH stretching, aliphatic CH stretching, C=O group and structural unit respectively. In the FT-IR spectrum of chitosan from the cuttlebone of Sepia pharaonis showed the band at 3355.34 cm^{-1} corresponds to H-bonded NH_2 and OH stretching, as indicated in Fig. 5. The band at 2890.13 cm^{-1} corresponds to aliphatic C–H stretching. The band at 1658.67 cm^{-1} corresponds to the amide stretching of C=O. Six bands at 1560.30, 1379.01, 1157.21, 1075.24 and 896.84 cm^{-1} correspond to the structural unit of chitosan.

3.2.2. X-ray diffraction (XRD)

The X-ray diffraction pattern of cuttlebone from S. pharaonis in comparison with reported cuttlebone (Weaver et al., 2011), was shown three main peaks at 27°, 34° and 47° of 2θ as shown in Fig. 6. The Sepia pharaonis chitin has four peaks at 2θ=11, 26, 34 and 47 as shown in Fig. 7. The Sepia pharaonis chitosan has four peaks at 2θ=10.5, 19.5, 29.9 and 34.5 as shown in Fig. 8. The S. pharaonis cuttlebone, chitin and chitosan XRD patterns were also similar to their corresponding standard patterns (Ramasamy et al., 2014).

3.2.3. Viscosity measurements

Many authors are of the opinion that the molecular weight of chitosan has an important effect on its activity. Viscosity average molecular weight (using Ostwald viscometer) the commercial chitosan from China and Sigma product showed the molecular weight ranged between 213 kDa and 549 kDa. In this research work, the molecular weight of *S. Pharaonis* chitosan was calculated as 350.06 kDa which is comparatively lower than that of the above mentioned crab chitosan. The variation in molecular weight may be due to the difference in deacetylation degree and the source of chitosan (Ramasamy et al., 2014).

3.3. Preparation of some acyl-chitosan derivatives

Synthesis of acyl-chitosan derivatives was carried out according to general reaction of amine and acyl chloride condition as shown in Fig. 9. The intermediate acid chloride was prepared from the

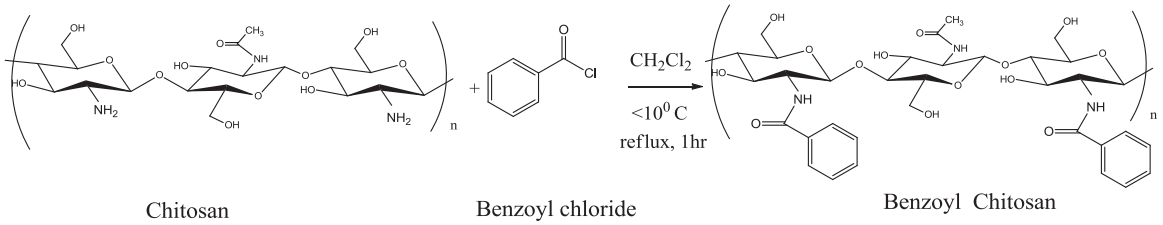

Fig. 10. Preparation of acyl-chitosan derivatives.

Table 1
Reaction of chitosan with acyl chlorides.

Entry	Acyl chloride	Product	Decomposition point (°C)	Yield (%)
1	Benzoyl chloride	Benzoyl chitosan	175	87
2	2-Hydroxy benzoyl chloride	2-Hydroxy benzoyl chitosan	195	90
3	4-tert-Butyl benzoyl chloride	4-tert-Butyl benzoyl chitosan	185	96
4	4-Chloro benzoyl chloride	4-Chloro benzoyl chitosan	172	98

reaction of carboxylic acid with $SOCl_2$ in dry media. The acylation of chitosan was performed in two steps: activation of chitosan and the acylation reaction (Tien, Lacroix, Ispas-Szabo, & Mateescu, 2003).

In this study for synthesis of acyl-chitosan derivatives, various benzoyl chloride derivatives were reacted with chitosan, as shown in Table 1.

3.3.1. Selected spectral data

3.3.1.1. FT-IR spectrum of 4-chloro benzoyl chitosan. The FT-IR spectrum of the 4-chloro benzoyl chitosan showed bands of 3534.95, 3072, 1686, 1621 cm^{-1}, and 648 cm^{-1} indicating the H-bonded NH and OH, aromatic C–H, C=O, aromatic C=C stretching respectively. The peak of 1419 corresponded to structural unit stretching as shown in Fig. 10.

3.3.1.2. X-ray diffraction (XRD) 4-chloro benzoyl chitosan. The X-ray diffraction pattern of 4-chloro benzoyl chitosan from S. pharaonis was shown three main peaks at 12°, 20.5° and 29.5° of 2θ as shown in Fig. 11.

3.3.1.3. Elemental analysis (C, H and N) 4-chloro benzoyl chitosan. The carbon, hydrogen, nitrogen and chlorine of 4-chloro benzoyl chitosan obtained from S. pharaonis found 52.08%, 5.24%, 5.37%, 12.20% respectively as shown in Fig. 12.

4. Conclusion

β-chitin have been isolated from Persian Gulf S. pharaonis cuttlefish bone, by treatment with dilute NaOH solution for deproteiniztion, dilute HCl solution for demineralization and dilute NaOCl solution for decolorization. In a later step, β-chitin was deacetylated by concentrated NaOH solution to afford β-chitosan with good to high yields. Finally, acyl chitosan derivatives were prepared by the reaction of β-chitosan with corresponding acyl chlorides. The yields, structural and spectral properties of compounds have been studied using FT-IR, XRD, DDA and CHN

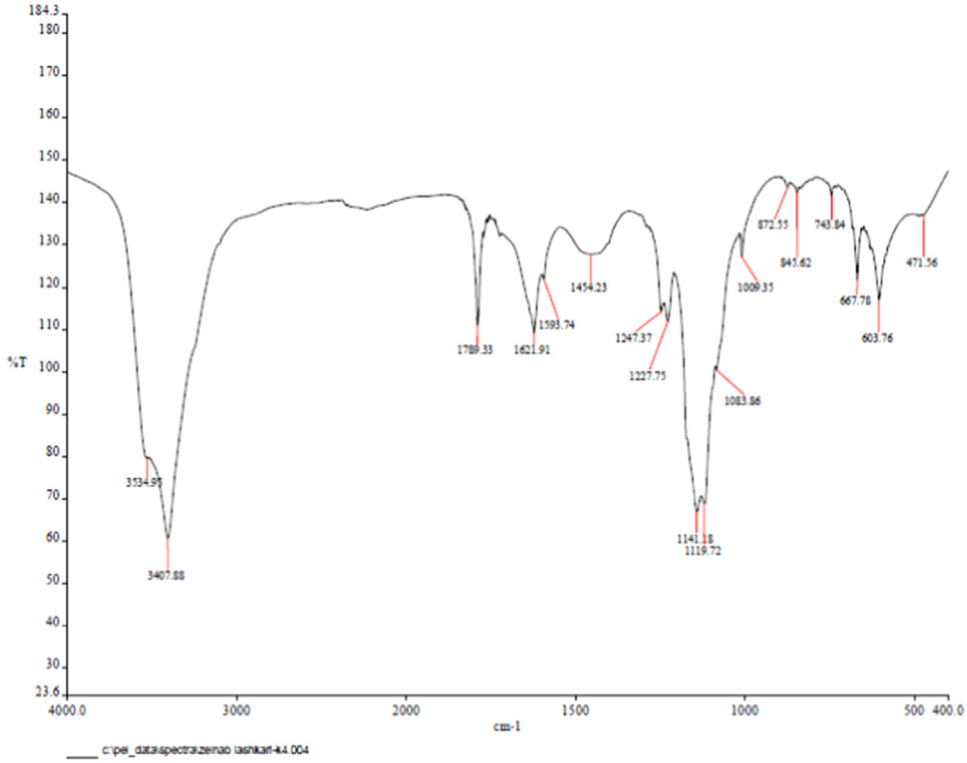

Fig. 11. FT-IR spectrum of 4-chloro benzoyl chitosan.

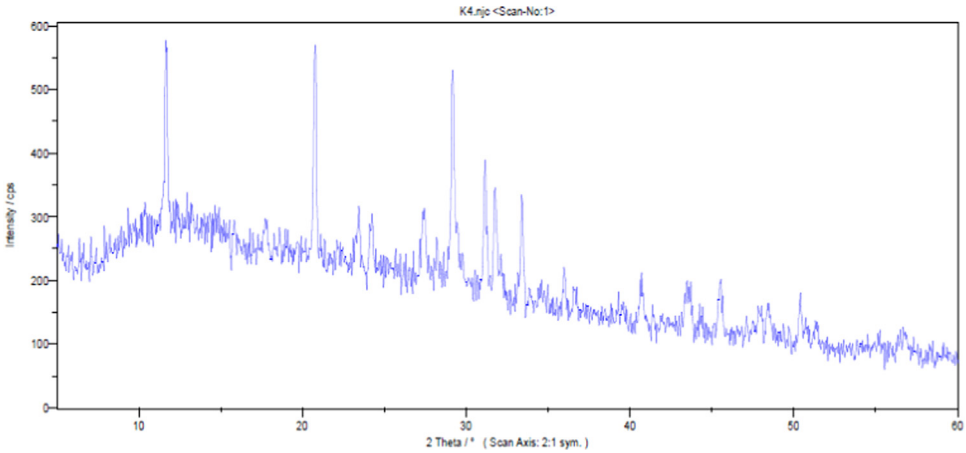

Fig. 12. XRD 4-chloro benzoyl chitosan.

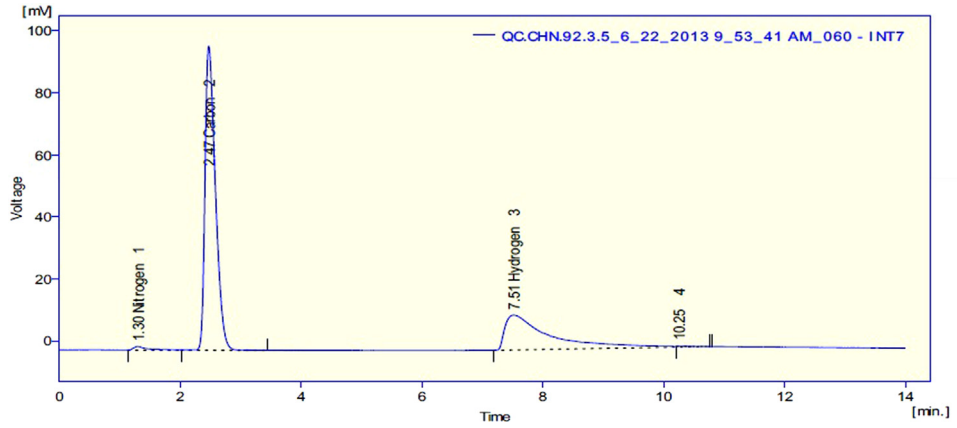

Fig. 13. Elemental analysis of derivation 4-chloro benzoyl chitosan.

methods. The degree of deacetylation was calculated as 90% (Fig. 13).

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