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Extraction and characterization of ß-chitin from sardine's scales *Sardina pilchardus (Walbaum, 1792)*

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Abstract

Sardine (*Sardina pilchardus*) is a common Mediterranean pelagic fish known as an important source of marine products in Morocco. In parallel, the high processing of sardines produces a huge quantity of sardine scales that causes environmental problem. Using the sardine scales for the production of chitin can enhance the economic value of sardine factories, and can reduce their adverse impact on nature. This study aimed to extract and characterize chitin for the first time from sardine scales basing on demineralization and deproteinization. The physicochemical structure of chitin extracted from sardine scales was determined by FT-IR, TGA, XRD, SEM, and EDAX. FTIR analysis revealed the β-form of the chitin extracted. The molecular weight (Mw) and the degree of Acetylation (DA) obtained were about 145KDA and 76% respectively. TGA determined thermal stability for the obtained chitin, which ranged between 150 to 250°C. XRD showed the amorphous structure of ß-chitin extracted and the crystalline index value of the chitin extracted (CrI) was 68%. While SEM and EDAX exposed that the chitin extracted has characterized by long chains and high purity. The results obtained in this current study showed that *Sardina pilchardus* can be used as a new alternative source of chitin..

Keywords: Chitin, Sardine scales, Demineralization, deproteinization, physicochemical properties.

1. Introduction

Chitin ($C_8H_{13}O_5N$) n is a copolymer of N-acetyl-D-glucosamine units linked with ß-(1-4) glucosidic bond, where N-acetyl-D-glucosamine units are predominant in the polymeric chain; it is the most abundant polysaccharide in nature after cellulose[1] . Chitin is naturally occurring renewable and biodegradable polysaccharide, non-toxic, odorless, physiologically inert and mechanically stable polymers. The extraction of chitin can be done by the biological or chemical method [2]. Depending on the source, chitin has three polymorphic forms: alpha (α), beta (β) and gamma (γ) [3]. Chitin is the important component of several living organisms as green algae [4], fish scales[5], crabs[6], squid cartilage[7], shell shrimps[8], outer cover of insects[9, 10], and other taxonomic groups. Alpha chitin is obtained from the fungal cell membrane and shell of crustaceans such as crabs and shrimps. Beta chitin derived from diatoms and squid arms. However, there is a small percentage of natural gamma chitin as a combination of alpha and beta [11]. The annual production of chitin is approximatively about 10^{12}-10^{14} tons/year, most of it is produced from marine resource [12]. Potential and usual applications of chitin are estimated to be more than 200 [13-17].Chitin is ordinarily insoluble in water and many organic solvents [14]. Chitin can be solubilized in some solvents as hexafluoroisopropanol, hexafluoroacetone, and chloroalcohols in conjunction with an aqueous solution of mineral acids, and dimethylacetamide containing 5% lithium chloride [15]. Nowadays, many studies have been demonstrated the possibility of extracting chitin and chitosan from fish scales [5, 20-24]. Fish scales represent approximatively 2% of total residues and it has no nutritional value in a raw state [18]. Nevertheless, fish scales are recognized by their wealth in biomaterials of high added value as collagen [19], hydroxyapatite[27- 29] , gelatin [30- 32]. In Morocco, the strong processing of sardines (*Sardina Pilchardus*) generates considerable tonnage of scales, which are not valued. That marine waste causes a crucial environmental problem that must be resolved as much as possible to decrease its impact on the environment. In this study, the abundance of sardine scales is one of the reasons for its use; this raw material can be recovered namely from ports, sardines canneries or restaurants. On the other hand, the direct rejection of sardine scales in fish processing industries or nature causes a serious problem of waterlogging and undergoes a rapid enzymatic and bacteriological process. Consequently, the extraction of chitin from fish scales will be among bio-innovations and eco-friendly solutions, and it will be also a great advantage for fish Moroccan processing industries as an alternative source for people allergic to chitin extracted from shrimp shell [24]. The present research aimed to extract for the first time chitin from sardines scales (*Sardina Pilchardus*), basing on the chemical method. Then, the obtained chitin was characterized by employing different techniques such as FT-IR, TGA, XRD, SEM, and EDAX. Physico-chemical properties of chitin extracted were also determined.

2. Material and Methods

2.1. Pretreatement of the raw material

Experiments were performed at the Laboratory of Biotechnologies in the Specialized Center of Valorization and Technology of Sea Products National Institute of Fisheries Research (INRH) of Agadir. Fish (*Sardina pilchardus*) scales were obtained from the port of Agadir in fresh conditions then washed thoroughly with chilled tap water to remove adherent proteins, organics and other impurities attached to the surface. Then, the washed scales are dried, placed in plastic bags under vacuum at room temperature and stored until use.

2.2. Extraction of chitin

Figure.1 displays the schematic flow diagram elucidating different steps in extraction of chitin from sardine scales. The extraction method of chitin from sardine scales requires two essential steps as demineralization and deproteinization. In this research, the extraction method of chitin was based referring to the protocol reported by [24] with slight modification. Briefly, sardine scales (*Sardina Pilchardus*) were dissolved in the 0.5M HCl 1:20(w/v) ratio for 60min under constant stirring (750 rpm) in an ambient temperature. Then, rinsed several times until neutrality with

distilled water to eliminate the excess HCL existing in the treated product, and finally dried at 30°C for 12h. The demineralized scales are cleaned from remaining proteins with sodium hydroxide using 100ml of 1% NaOH aqueous solution. The reaction time was about 180 min at 50°C under constant stirring (250rpm). The residue obtained was washed with distilled water to attain neutrality and then oven-dried at 30°C for 12h. The product obtained from demineralization and deproteinization of sardine scales was chitin. At this stage, chitin extracted is white and it does not require any bleaching step.

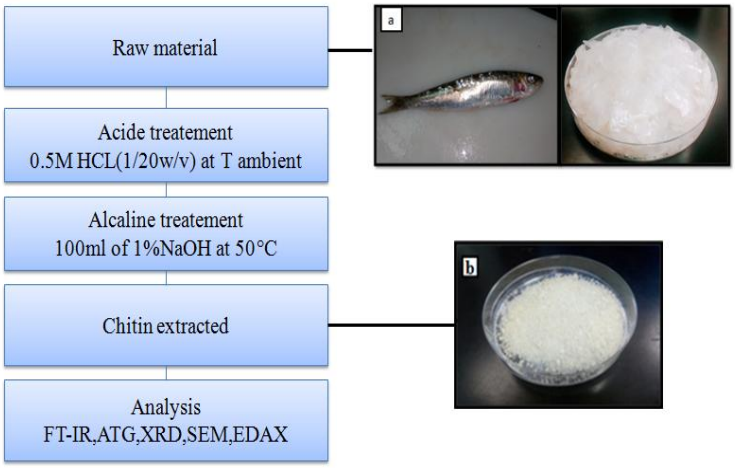

Figure 1: (a) Raw material, (b) chitin extracted from sardine scales

2.3. Chemical reagents

Hydrochloride acid (35-38%) CAS: 7647-01-0, and Sodium hydroxide pellets (98%) CAS: 1310-73-2 were purchased from Loba Chemie Company; all chemical reagents were analytical grade, without further purification.

2.4.Physico-chemical characteristics

2.4.1. Determination of Ash, moisture, protein.

The ash content of chitin sample was determined using gravimetric method [25]. The moisture content was measured according to [26]. Nitrogen content was determined by the Kjeldahl method (AOAC method 984.13).

2.4.2. Solubility of chitin.

Chitin solubility was determined according to the study in [27], by placing 0.1 g of chitin into a pre-weighted centrifuge tube, followed by dissolution in 10 ml of 40% aqueous acetic acid at room temperature under constant stirring for 30 minutes using an incubator shaker which was running at 240 rpm at 25 °C. The percentage of solubility was calculated using the following equation:

$$\text{Solubility\%} = \frac{(M1-M2)}{(M1-M0)} \times 100 \quad (1)$$

Where M_0 is the initial weight of tube, M_1 and M_2 are the initial weight of the tube +sample and the final weight of the tube sample, respectively. All physicochemical experiments were repeated in triplicate and the mean was taken.

2.4.3. Molecular weight of chitin extracted

The molecular weight (M_W) of chitin extracted from sardine scales was determined viscometrically. The intrinsic viscosity of chitin extracted was determined by an Ubbelohde viscometer at 20◦C. The average molecular weight was calculated referring to the Mark –Houwnik Equation , the procedure was described in [28]:

$[\mu]=K.M^{a}$ (2) Where [µ] is intrinsic viscosity of chitin obtained. M is the average molecular weight of ß-chitin, α is 0.68 (solvent NaOH 10 wt. %), and K is 0.1.

2.5.Structural Characterization of chitin extracted

2.5.1. Fourier transforms infrared spectroscopy (FTIR).

Chitin obtained is measured from the infrared spectrum recorded on Vertex 70 series FT-IR spectrum. The transmittance was measured as a function of the wave number between 4000 and 400 cm^{-1}, with solution of 4 cm^{-1} and the number of scans equal to 32. Chitin dried was thoroughly mixed with Kbr (1mg of sample mixed with 99 mg of Kbr), then the dried mixture was pressed to obtain a uniform film with in a homogeneous sample disk. The degree of acetylation (DA %) of chitin was calculated according to the formula quoted in[29] :

$$DA\%=100-[(\frac{A1655}{A3450}\times\frac{100}{1,33})] \quad (3)$$

Where, A_{1655} was chitin absorbance at the 1655cm^{-1} wavelength , and A_{3450} was chitin absorbance at the 3450 cm^{-1} wavelength.

2.5.2. Thermal properties (TGA).

Thermogravimetric analysis (TGA) of chitin extracted was performed to estimate their thermal stability and degradation profiles, using thermal gravimetric analyzer (Labsys Evo TGA STA-EGA). 1mg of chitin extracted was weighed and heated at a constant heating rate of 10°C/min from10 to 600°C and under a nitrogen flow.

2.5.3. Scanning electron microscopy and energy dispersive X-ray spectroscopy (SEM/EDAX).

A scanning electron microscope (SEM) was conducted using a Tescan Vega 3 operated at 10kV to examine the surface morphology structure of fish scales and chitin extracted respectively. The dried samples were ground and coated with carbon under vacuum using a sputter coater. Energy-dispersive X-ray spectroscopy (EDAX) was used to determine the elemental composition of the raw material and chitin extracted respectively.

2.5.4. X-ray powder diffraction (XRD).

X-ray diffraction (XRD) was used to determine the crystallinity index of the chitin extracted.

The X-ray diffraction analysis of chitin extracted were recorded using Smart Lab X-ray diffractometer with Cu radiation (k=40 kV, 30mA).The measurements taken were in the scanning between 2θ angles of 5-70 at a scanning speed of $50s^{-1}$[30]. The crystalline index was calculated as follow:

$$\text{Crystalline index (\%)} = \frac{I110-Iam\times100}{I110} \quad \textbf{(4)}$$

Where I_{110} is the maximum intensity of the (110) diffraction peak at 2θ = 20°.

I_{am} is the intensity of amorphous diffraction at 2θ = 16°.

3. Results and Discussion

3.1. Chemical composition of the chitin obtained from sardine scales

The extraction method of chitin from sardine scales was done basing on the protocol of [24], excluded from the bleaching step. The extraction of chitin is based on two main stages demineralization and deprotenization. Demineralization step requests elimination of minerals, primarily $CaCO_3$.The process of deprotenization is very important step, which aimed to remove proteins attached to chitin. Table 1 depicted the proximate composition of chitin obtained. After demineralization (DM) and deproteinization (DP) chitin obtained is characterized by its whitish color. In this study, the chitin extracted does not undergo any bleaching agent, because the raw material does not contain any pigments like shrimp shells which are rich in ß-carotene. Moorjani et al in[31] showed that the bleaching step is not obligatory because it decreases the viscosity of chitin. The yield of chitin obtained was found to be 10.38% on dry basis weight, which is lower of that obtained from common carp, Nile Tilapia, and shrimps [16, 24 , 8]. We might mention that sardine scales have a transparent, soft and small scales comparing with those of Nile tilapia and common carp. However, this yield obtained is very important considering the availability of the raw material during

the year and its low price. According to the study of Kaya et al in [32], the percentage of chitin from shrimps, crabs, krill vary between 20% and 30%. While, the percentage of chitin obtained from insects ranged from 10% to 36%. The yield of chitins obtained is affected by several parameters like the species studied [33], the isolation method used[34], and the extraction method [42, 43]. The chitin moisture content is approximatively similar to the chitin obtained from Labeo Rohita[37], and mussel shell [38]. The ash content of the chitin extracted from sardine scales is absent, this result revealed the highest grade of the chitin extracted, and showed the effectiveness of the method adopted for the elimination of $CaCO_3$ [39]. The nitrogen content in the chitin extracted was about 6.70%, which is near to the theoretical rate of 6.9% for pure chitin. This result is in agreement with the study of [40]. The obtained result is approximatively near of the nitrogen rate for common carp [16]. The percentage of solubility of the chitin treated with 40% of acetic acid was about 95%, which shows a high solubility of chitin extracted from sardine scales comparing with those extracted from Pang scales and silver scales which represent a value of 68% and 67.54% respectively[17]. The existence of proteins and impurities in chitin inhibits solubility, which is explained by the strong inter and intramolecular bonds existing at the hydroxyl and acetamide groups [41]. The high rate of solubility could be explained also by the absence of the ash [39], and can be one of the advantageous features in chitin formulations applications. The molecular weight (Mw) of chitin obtained from sardine scales was 145KDa (Table1). This value classifies chitin extracted from sardine scales in the range of the medium molecular weight [42].This obtained value is very encouraging which will allow the application of chitin in different fields. The low solubility of chitin makes difficult the determination of molecular weight[43].Thus, the grinding can influenced the Mw of chitin obtained [44]. According to the obtained results, Sardine scales are therefore assuring an alternative source of chitin.

Table 1 Proximate composition of chitin extracted from sardine scales on dry basis at 25°C.

Properties	**Chitin extracted**
Moisture *(%)	12.00±0.50
Ash* (%)	Nd
Nitrogen*(%)	6 .70±0.10
Appearance	White
Solubility in acetic acid (%)	95±0.00
Yield*(%)	10.38±0.10
DA(%) by FT-IR	76±0.00
M_W (KDa)	145±0.02

*Results are means triplicate determination ± standard deviation

3.2. Fourier Transform Infrared Spectroscopy (FT-IR) of chitin

The IR spectra of chitin extracted from sardine scales are shown in figure 2. According to the FT-IR spectrum (Fig. 2), the chitin extracted have a band appeared in 3435 cm^{-1} represents the stretching vibration of aliphatic (O- H), the absorption peak at 2935 cm^{-1} indicates the (C-H) vibration of (CH3). The absorption band at 1650 cm^{-1} is the stretching vibration of the carbonyl group, C=O from acetamide. The form alpha and beta of chitin extracted can be distinguished using FTIR analysis. In the ß-chitin, just one band can be observed at 1656 cm^{-1} [5, 44,46].Referring to the FT-IR spectrum of chitin extracted from sardine scales, it was proved that chitin obtained is in ß-form. The results obtained are in line with that obtained for Common carp[16]. .The degree of acetylation (DA %) of chitin is an important parameter because it influences all physico-chemical properties of the polymer. Theoretically DA value of the pure chitin is known 100% [48]. In this study, the value of DA of the chitin obtained from *Sardina Pilchardus* scales was found to be 76.06% (table1), which is higher than DA of chitin obtained from Pang scales and Silver scales [17]. Among the benefits of ß- Chitin is the high reactivity and affinity with solvents[49].

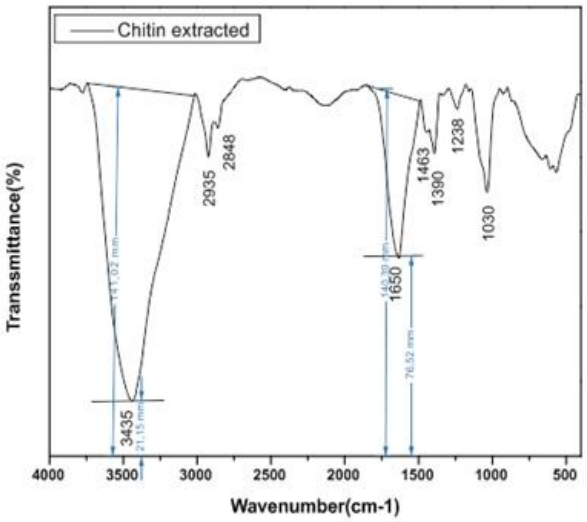

Figure 2: FTIR spectra of the chitin extracted from sardine scales (Sardina Pilchardus)

3.3. Thermogravimetric analysis (TGA)

The thermogravimetric curve of chitin extracted from sardine scales is shown in figure .3.According to the same curve the mass loss of the chitin extracted from sardine scales took place in two stages. Those findings are similar to results reported by other researchers [37, 39, 48]. The first mass loss for chitin extracted is about 12%, which varied between 50 to 150°C due to the evaporation of water free and hydrogen-bonded of water to the chitin structure. The second mass loss is around 50%, which ranged between 250°C-420° C. This attributed to the decomposition of the polymer. The comparison between the thermal degradation of several species shows that the difference between chitins of various sources of origin remains small. Except that alpha chitin is more stable because of its orthorhombic form and beta chitin remains in less stable because of its monoclinic form [45, 50]. The study was done by the team of Kaya et al in [32] shows that the rate of loss mass and the interval of temperature differed up on source origin. According to figure 3, the thermal stability of chitin obtained from sardine scales is less than that of shrimps and nearly close to the thermal stability of squid and Labeo Rohita [37,49].Consequently, the thermal stability of chitin extracted from sardine scales orients the use of chitin in the field of agriculture, food industry and cosmetic.

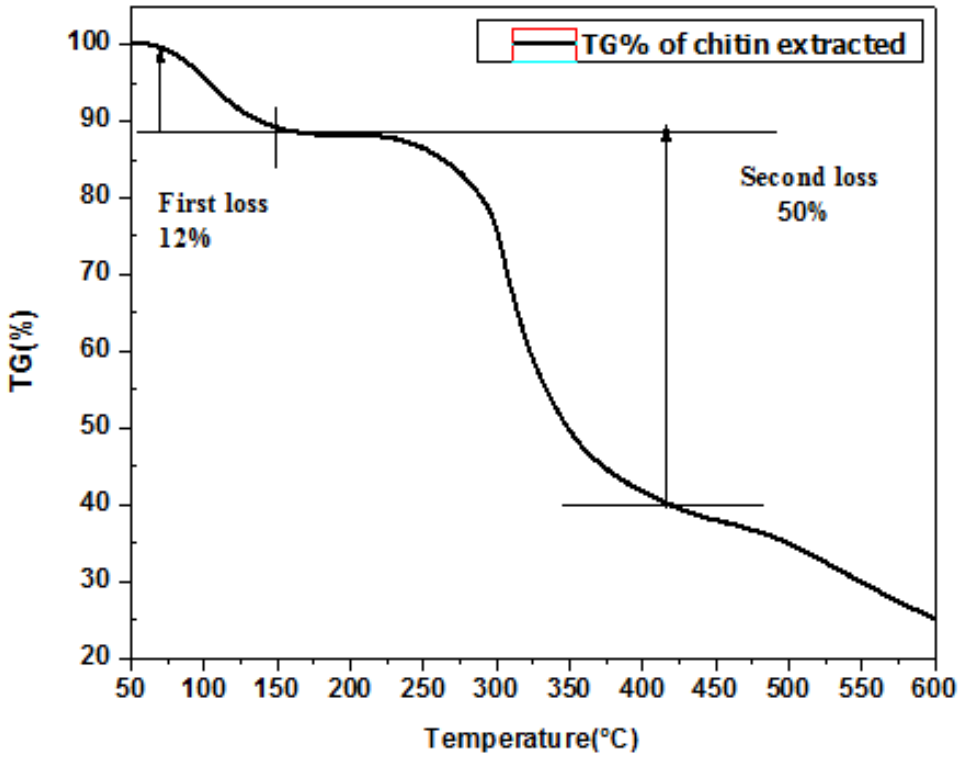

Figure 3: TGA of the chitin extracted from sardine scales (Sardina Pilchardus).

3.4. Crystalline structure of chitin extracted.

The X-ray diffraction of chitin obtained from sardine scales is represented in figure 4.The XRD patterns of chitin extracted showed two specific sharp peaks at 2θ between 5-60° around 9.1, and 20.3°. The extracted chitin in the current study has both amorphous and crystalline regions in the structure, this structure have been reported from anterior works on common carp [16,50].The CrI value of chitin obtained from sardine scales(*Sardina Pilchardus*)was calculated to be 68 %.The CrI values of chitins differs by the groups of organism and the isolation method used.[48]. Previous studies showed that ß-chitin exhibited two sharp peaks at around of 7.9-9.8, and19.3-20.3[28,51,52]. The obtained result confirms the beta form of chitin extracted from sardine scales.

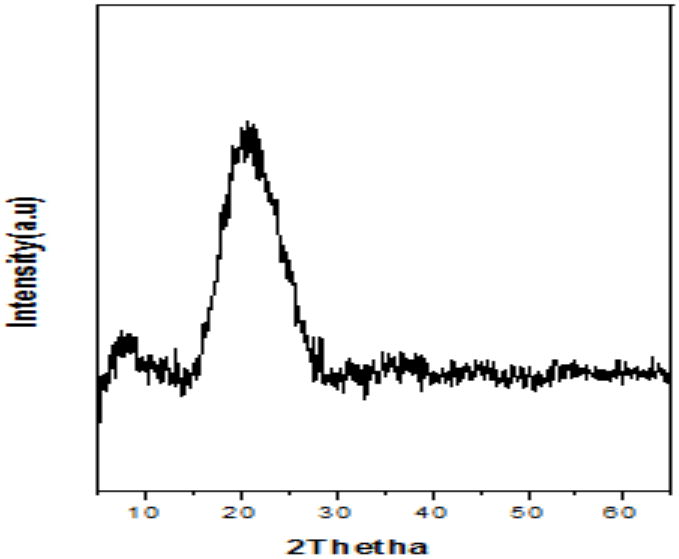

Figure 4:X-Ray diffraction patterns of the chitin extracted from sardine scales (*Sardina Pilchardus*)

3.5. Surface morphology of sardine scales (a), and chitin extracted (b)

The surface morphology of sardine scales (a) and chitin extracted were shown in figure 5. The structure of ground sardine scales is characterized by a filamentous appearance, which is formed of proteins, minerals traces of lipids and chitin. After demineralization and deproteinization, the surface morphology of chitin extracted from sardine scales is characterized by its non-porosity and non-regularity. The same surface morphology of chitin was reported by Kaya et al in [32], who have categorized the surface morphology of chitin in four groups. Our chitin is categorized into a form that contains fibers and no pores. This result confirmed the non-crystallinity of chitin extracted mentioned in figure 5.

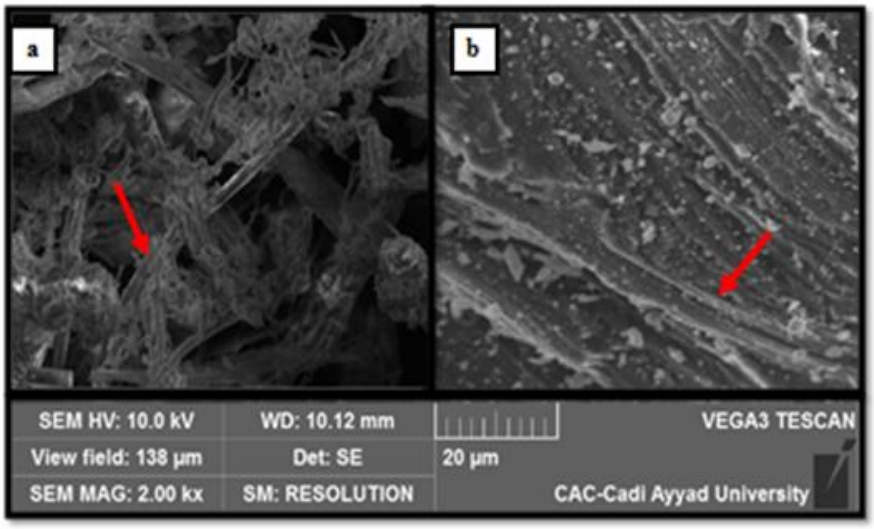

Figure 5: SEM photographs of the raw material (a) and ß chitin extracted (b)

3.6. EDAX spectra of sardine scales (a) and chitin extracted (b)

The chitin obtained from sardine scales *Sardina pilchardus* is selected for examination by Energy Dispersive X-ray Spectroscopy (EDAX) analysis. According to figure 6, sardine scales (a) are characterized by several elements with high level such as calcium, oxygen, carbon, phosphorus, and sodium. However, after demineralization (DM), and deproteinization (DP) the obtained chitin (b) is free of mineral elements. The current result confirms the efficiency of the extraction system and proves the high purity of the chitin isolated from sardine scales. The absence of mineral part in chitin extracted (b), is confirmed by the absence of ash mentioned in table 1.

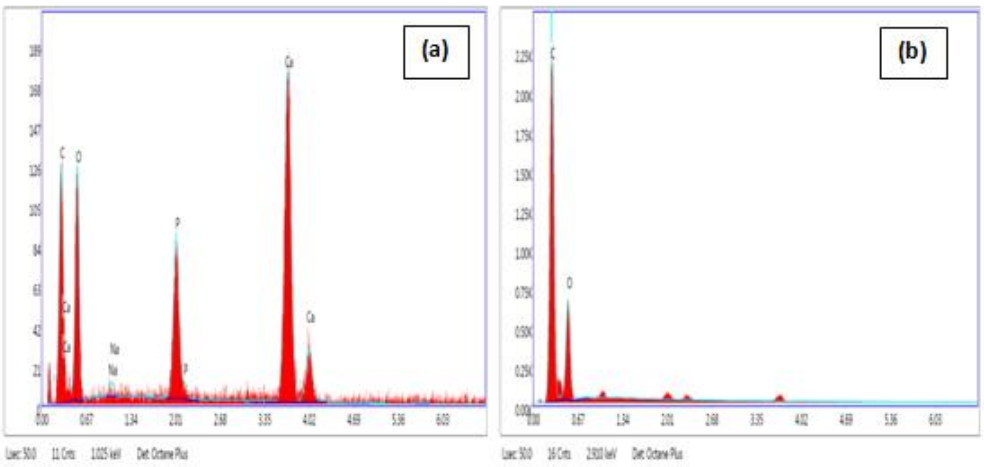

Figure 6: EDAX spectra of raw material (a), and chitin extracted (b)

4. Conclusion

Chitin has been extracted from *Sardina pilchardus* of the Moroccan littoral, basing on demineralization and deprotenization steps. *Sardina pilchardus* scales chitin was characterized based on FT-IR, TGA, XRD, SEM, and EDAX. The results obtained confirmed that the chitin extracted from sardine scales has ß-form. Referring to this study, it may be suggested that chitin extracted from sardine scales can be used for the food industry, agriculture, medicine, and pharmacy. This new study proved firstly that scales of *Sardina pilchardus* will be an alternative source of chitin, due to the abundance of the raw material throughout the year, and the good purity of the chitin obtained. On the other hand, the valorization of sardine scales will decrease the environmental pollution in Moroccan littoral and will improve the profit margin of the sardine processing factories.

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