Chitin Preparation by Demineralizing Deproteinized Lobster Shells with CO_2 and a Cationite

Miguel Ángel Ramírez[1], Patricia González[2], Juan Reinerio Fagundo[2], Margaret Suarez[3], Clara Melian[3], Tania Rodríguez[1] and Carlos Peniche[4*]

[1]*Los Palacios Unit (UCTB), National Institute of Agricultural Sciences (INCA), Mayabeque, Cuba*
[2]*Faculty of Chemistry, University of Havana, Havana, Cuba*
[3]*Faculty of Radiochemistry, Higher Institute of Technologies and Applied Sciences (InSTEC), Havana, Cuba*
[4]*Biomaterials Center (BIOMAT), University of Havana, Havana, Cuba*

Received May 24, 2016; Accepted July 14, 2016

ABSTRACT: The inorganic components of crustacean shells are usually removed using HCl solutions. This provokes undesirable modifications in the extracted chitin. In the present procedure, deproteinized lobster shells were demineralized with CO_2 and a cationic resin (cationite). The resulting chitin (CHI-CO_2) is compared in terms of degree of acetylation (DA), crystallinity index (CrI) and thermal stability with chitins obtained by demineralization procedures with HCl (CHI-HCl) and ethylenediaminetetraacetic acid (CHI-EDTA). The ash content of chitins demineralized with CO_2 was similar to that of chitins prepared using HCl or EDTA. However, the resultant DA and CrI of CHI-HCl and CHI-EDTA were lower than those of CHI-CO_2. Thermal stability of CHI-CO_2 was also higher, with maximum decomposition velocity at 360 °C, above those of CHI-EDTA (348 °C) and CHI-HCl (332 °C). This indicates that the use of CO_2 and a cationite for demineralizing lobster shells is a promising alternative to conventional HCl and EDTA treatments for obtaining chitin.

KEYWORDS: Carbon dioxide, chitin, demineralization, lobster shells

1 INTRODUCTION

Chitin is a linear polysaccharide composed of *N*-acetyl-2-amino-2-deoxy-D-glucose units linked together by β(1→4) glycosidic links. It is widely distributed in nature where it is found in arthropods, insects, arachnids, fungi and algae, among others [1].

Commercial chitin is generally produced from the residues of seafood processing plants, the main sources being the shells of shrimps, prawns, crabs and lobsters. Although the composition of the raw material varies with the species, the extraction procedures almost invariably contain four main steps: raw material conditioning (cleaning and grounding), protein extraction (deproteinization), elimination of inorganic material (demineralization) and bleaching (pigment removal) [2–4]. Most of the techniques developed involve chemical processes to remove the protein and the inorganic material and some include a bleaching step of the resulting chitin either by solvent extraction or by oxidation of the remaining pigments. These chemical isolation treatments can generate undesirable modifications in the resulting chitin, such as decrease in the degree of acetylation (DA), crystallinity, molecular weight and thermal stability. Enzymatic treatments are being investigated as a promising alternative. Processes have been reported using enzyme extracts or isolated enzymes and microbial fermentation, but still without the efficiency of chemical methods, in particular as regards the removal of the inorganic material [5].

Deproteinization of crustacean shells is often carried out with dilute NaOH solutions at 65–100 °C from 0.5 to 72 h. Some procedures involve two consecutive treatments for a short time, since long times or high temperatures can provoke chain scission and partial deacetylation of the polymer [2]. Enzymatic extracts or isolated enzymes and microbiological fermentation have been tested with some success, but the alternative of the enzymatic/microbiological treatment, besides being time-consuming, usually leaves 1–7% of residual protein [5].

The main inorganic component of crustacean shells is $CaCO_3$, which is usually eliminated using dilute HCl

**Corresponding author*: peniche@fq.uh.cu

DOI: 10.7569/JRM.2016.634121

solutions at room temperature, although other acids have also been used (HNO_3, H_2SO_4, CH_3COOH). The acid concentration and the time of treatment depend on the source of chitin, but, in any case, high temperatures should be avoided to prevent polymer degradation [2]. Alternative treatments to prevent degradation consist in the use of ethylenediaminetetraacetic acid (EDTA) [6] or ionic liquids [7].

In a recent publication we used CO_2 for the demineralization of lobster shells and described the kinetics of the process, which turned out to be of pseudo-first-order [8].

The aim of the present work is to devise a method of obtaining chitin using milder conditions in the demineralization step than the traditional industrial processes using HCl solutions. In the present procedure, demineralization of deproteinized spiny lobster shells will be performed using CO_2 and a cationic exchange resin. The resulting chitin will be compared in terms of degree of acetylation (DA), crystallinity index (CrI) and thermal stability of chitins obtained by demineralization using HCl and EDTA treatments.

2 EXPERIMENTAL

2.1 Materials

Spiny lobster (*Panulirus argus*) shells were obtained from the seafood processing plant at La Coloma (Pinar del Rio, Cuba). The cation exchange resin (hereinafter also called cationite) used was Dowex 50X8, Na^+-form (BDH, England). CO_2 gas cylinder with 99% purity and 10 MPa was supplied by the CO_2 plant (Guanabacoa, Cuba). All other reagents used were analytical grade.

2.2 Lobster Shell Processing Before Demineralization

Lobster shells were washed thoroughly with abundant water to remove adventitious proteins, soluble organics and other impurities, and dried in air for 24 h. The dry shells were ground in a hammer mill to particle sizes between 200 and 400 µm. Afterwards, they were deproteinized by three successive 1 hour treatments with 0.5 M NaOH at 80 °C. Next they were washed with tap water and distilled water until neutral, dried in air and stored at 4 °C until use.

2.3 Demineralization by Conventional Methods

Chitin samples were obtained by two widely used demineralization procedures [2]. One sample was obtained with HCl, and the other with ethylenediaminetetraacetic acid (EDTA). They were labeled as CHI-HCl and CHI-EDTA, respectively. CHI-HCl was prepared by treating 50 g of dry deproteinized lobster shells with 2 M HCl at 30 ± 1 °C and a solution to solid ratio of 10 $mL \cdot g^{-1}$. After 3 h, the resulting chitin was removed by filtration, washed with abundant water to neutrality and dried in an oven at 50 °C. CHI-EDTA was prepared by treating 50 g of dry deproteinized lobster shell with 500 mL of 1 M EDTA in NH_4Cl/NH_3 buffer solution at pH 10 for 24 h. After that, the material was removed by filtration, washed with abundant water to neutrality and dried in an oven at 50 °C. Both chitin isolation procedures were made in triplicate.

2.4 Demineralization Using CO_2

Demineralization reaction was carried out in a 2 L Büchner funnel with a sintered glass disc No.1 connected with a hose to a CO_2 gas cylinder provided with a pressure regulator. This kind of arrangement has been used before for reactions involving CO_2 and it allows achieving good homogeneity when bubbling the gas through aqueous solutions [9]. CO_2 was supplied continuously at 5 $L \cdot min^{-1}$. Before reaction, 1 L of water was added to the funnel and was saturated with CO_2 bubbling the gas during 10 minutes. Then, a given amount of deproteinized shell was added and the CO_2 stream was maintained for 3 hours. The temperature was set at 25 ± 1 °C. Afterwards the resultant chitin (CHI-CO_2) was filtrated, washed with distilled water and dried in an oven at 50 °C. In order to ensure a high degree of demineralization a cationic exchange resin of Dowex 50X8 was incorporated into the reacting system. It was used in a 1:5 shell/resin mass ratio. The resin was placed in the reactor inside a polyethylene net to isolate it from the shell particles.

2.5 Characterization Techniques

Moisture content was determined by the gravimetric method. A previously weighed amount of sample was dried in an oven at 105 °C to constant weight. The moisture content was calculated from the difference between the weights of the original wet sample and the dry sample, as shown in Equation 1.

$$\text{Moisture content}(\%) = \frac{\text{Wet weight} - \text{Dry weight}}{\text{Wet weight}} \times 100 \quad (1)$$

To determine the ash content 1.0 g of sample was placed into a previously ignited, cooled, and tarred crucible. The sample was placed in a muffle furnace preheated to 600 °C for 4 h. The crucibles were allowed to cool in the furnace to less than 200 °C and then

placed into desiccators with a vented top. The ash content was calculated as follows:

$$\text{Ash}(\%) = \frac{\text{Weight of Residue}}{\text{Weight of Sample}} \times 100 \qquad (2)$$

Experiments were performed in triplicate and the results are expressed as the mean ± SD.

The HCO_3^-, Mg^{2+} and Ca^{2+} ion concentrations were estimated following recommended standard volumetric analytical methods established for their determination in water [10].

Protein contents were determined by micro Lowry method [11]. Protein was extracted from the shells with KOH 1 M with a liquid to solid ratio of 20 mL·g^{-1} at 90 °C with magnetic stirring for two h. After vacuum filtration, the protein content in solution was determined in a UV spectrophotometer. Bovine serum albumin (BSA) was used as standard. The content of lipids was evaluated by gravimetry after previous soxhlet extraction from the shell samples with n-hexane for 3 h.

Thermal behavior of samples was examined by differential thermal analysis (DTA) and thermogravimetric analysis (TGA) using a thermal analyzer NETSZCH SDT Q600 V8.3 at a heating rate of 10 °C·min^{-1}, from 25 to 900 °C in nitrogen atmosphere.

X-ray diffraction data were recorded in a PANalytical X'Pert Pro MPD (Almelo, The Netherlands) device equipped with Cu X-ray tube and two goniometers in θ–2θ vertical configuration and optical Bragg-Brentano inoLab®. Diffraction patterns were recorded over a 2θ range of 5–50° in continuous mode. The step size was 0.04° and 1.0 s per step.

The FTIR spectra were recorded by the KBr pellet technique in the spectral range from 4000 to 500 cm^{-1} by using a Bruker Tensor 27 (Bruker, Germany) Fourier-Transformed Infrared Spectrophotometer. Spectra were obtained with a resolution of 2 cm^{-1} and were averaged over 100 scans. Samples (5 mg) were thoroughly dried and ground with KBr. Discs were prepared by compression under vacuum.

3 RESULTS AND DISCUSSION

The main components of the shell of the spiny lobster *Panulirus argus* are listed in Table 1. It is worth mentioning the reproducibility of the composition found with the previously reported one and the high value of ash content of shells. This ash content is much higher than those reported for most chitin sources, such as the head of shrimps (20–30 wt%), squid pen (<0.7 wt%) or crab carapaces (40–58 wt%), depending on the species [3, 12]. However, it is similar to other lobsters, like *Homarus americanus* (>50 wt%) [13]. This implies that chitin isolation from spiny lobster will require harsher reaction conditions than most of typical chitin sources, such as a higher concentration of reactants and longer reaction times [2, 14].

Table 1 Main components of the shell of lobster *Panulirus argus*.

Component	Content (wt-%)	I. García (wt-%) [24]
Moisture	12.0 ± 0.5	11
Ashes	54 ± 2	52
Proteins	14 ± 1	15
Lipids	1.1 ± 0.7	n.d.
Chitin	16 ± 2	16

The data are expressed as the mean ± SD of three determinations. n.d.: not determined

The inorganic components of the spiny lobster shell have been evaluated by X-ray fluorescence [15]. Twelve different chemical elements were identified. The main ones were Ca (32.5 wt%), Mg (2.4 wt%), Cl (1.97 wt%), P (1.38 wt%) and Na (1.18 wt%). In spite of the high sensibility of the method, Cd, Pb, As and Hg were not detected.

The procedure followed to isolate chitin in the present study involved a protein extraction step preceding demineralization. Chitin samples obtained were not subjected to further purification steps before characterization, so that the difference between them would only be due to the procedure used for removing the inorganic component of the deproteinized shell.

The progress of the demineralization reaction of deproteinized lobster shells with CO_2 has been previously reported [8]. It was found that the pH of the starting CO_2 saturated solution was 4.5, but it increased rapidly upon the addition of the deproteinized shell, reaching a value of 6 after 50 min of reaction. The acidic conditions of this procedure are less severe than those used when demineralization is performed with HCl, where pH values lower that 2 are reached. These low pH values can provoke modifications in the native chitin, such as depolymerization and even some deacetylation [16].

The dissolution of calcium carbonate provokes a rapid increase in the concentration of Ca^{2+} and HCO_3^- ions in solution, which is accompanied by the increase in pH already mentioned. As expected, the ash content, determined at 600 °C, decreased continuously from 67 wt% for the starting material to 54 wt% after 50 minutes of reaction. However, afterwards no further decrease in the ash content was achieved.

This indicates that equilibrium is reached, impeding further demineralization [9]. The way to attain further decrease in the carbonate content of samples is to displace the equilibrium by removing the Ca^{2+} and Mg^{2+}

Table 2 Comparative results of demineralization experiments on deproteinized shells. Particle size, 300 μm; reaction time, 120 min; T = 25 °C.

Run	Ratio[1] ($g \cdot mL^{-1}$)	CO_2 Flux ($dm^3 \cdot min^{-1}$)	Resin (g)	$[Ca^{2+}]$ ($mg \cdot L^{-1}$)	$[Mg^{2+}]$ ($mg \cdot L^{-1}$)	$[HCO_3^-]$ ($mg \cdot L^{-1}$)	Ash[2] (wt-%)
1	8/800	5	0	326	44.4	1567	54 ±2
2	8/800	5	40	≤1.0	≤1.0	2013	1.3

[1]mass (g) of deproteinized shell/mL of solution.
[2]percent on dry basis.

Table 3 Humidity, ash and protein content of lobster shell and the chitins obtained by different procedures.

Composition (wt-%)	CHI-HCl	CHI-EDTA	CHI-CO_2
Moisture	10.6 ± 0.6	10.3 ± 0.5	10 ± 1
Ash	1.0 ± 0.3	1.2 ± 0.5	1.3 ± 0.6
Protein	<1%	<1%	<1%

ions from the solution. Consequently, the demineralization reaction was then carried out, introducing the cationic exchange resin Dowex 50X8 Na^+ in order to substitute the Ca^{2+} and Mg^{2+} ions in solution by Na^+ ions [8].

The effect of using the ion exchange resin on the extent of demineralization of a deproteinized shell having 67 ± 1 wt% ash content is appreciated in Table 2. When CO_2 is used alone (run 1), the ash content of the material remains very high (54 ± 2 wt%) even after 2 hours of reaction. At that time the Ca^{2+} and Mg^{2+} concentrations in solution reached 326 and 44,4 $mg \cdot L^{-1}$, respectively. When the resin is used (run 2) the Ca^{2+} and Mg^{2+} concentrations become lower than 1.0 $mg \cdot L^{-1}$ and the ash content of the resulting material is reduced to 1.3 wt%.

3.1 Characterization of Samples

3.1.1 Chemical Composition

The composition in terms of humidity, ash and protein content of the chitin samples obtained by demineralizing with HCl (CHI-HCl), EDTA (CHI-EDTA) and CO_2 (CHI-CO_2) are listed in Table 3.

The similar compositions of the three chitins can be appreciated. However, it has been stated that HCl can affect the structure of chitin [17] and hinder its use in certain applications [18]. On the other hand, the use of EDTA required a much longer time to achieve a satisfactory demineralization (12 hours). In this case, it was also necessary to considerably decrease the particle size and to increase the pH to 10 in order to obtain acceptable ash content. Taking into account this consideration, demineralization with CO_2 emerges as an attractive alternative.

It is worth mentioning that the yield of chitin calculated based on dry deproteinized lobster shells was

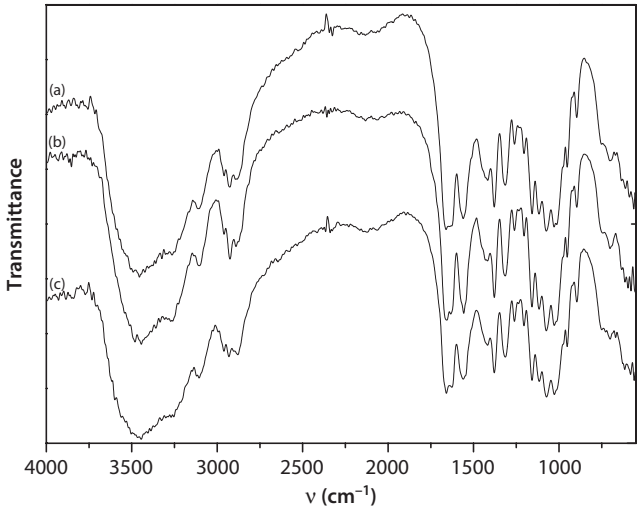

Figure 1 Infrared spectra of chitins: (**a**) CHI-EDTA; (**b**) CHI-EDTA; (**c**) CHI-CO_2.

almost the same for the three methods employed: 28 ± 1 for CHI-CO_2, 27 ± 0.5 for CHI-HCl and 28 ± 0.8 for CHI-EDTA. The data are the average value with the standard deviation of three different runs for each method.

3.1.2 Infrared Spectra of Chitins

The infrared spectra of the three chitin samples were very similar (Figure 1) and displayed the distinctive absorption bands at 2970–2874 cm^{-1} (aliphatic C–H stretching band), 1655 cm^{-1} (amide I), 1597 cm^{-1} (NH_2 bending) and 1320 cm^{-1} (amide III). The absorption bands at 1154 cm^{-1} (antisymmetric stretching of the C–O–C bridge), 1082 and 1032 cm^{-1} (skeletal vibrations involving C–O stretching) are characteristic of its saccharide structure [19].

The degrees of acetylation (*DA*) of the three chitins were evaluated from the infrared spectra using the equation of Baxter *et al*. [20] and a more recent one proposed by Brugnerotto *et al*. [21]. In Baxter's equation:

$$DA(\%) = \left(\frac{A_{1655}}{A_{3450}} \right) \times 115 \quad (3)$$

where A_{1655}/A_{3450} represent the absorbance ratio of the bands at 1655 and 3450 cm^{-1}, respectively. In contrast,

Table 4 Degree of acetylation of chitins calculated by IR spectroscopy and thermal analysis.

Sample	Degree of acetylation (DA)		
	Baxter (Eq. 3)	Brugnerotto (Eq. 4)	Thermal (Eq. 5)
CHI-EDTA	55.4	70.4	85.8
CHI-HCl	66.7	74.2	80.5
CHI-CO_2	72.6	88.6	92.9

the equation proposed by Brugnerotto makes use of the absorbance ratio of two other bands, the one at 1320 cm^{-1} and the one at 1420 cm^{-1}:

$$\frac{A_{1320}}{A_{1420}} = 0.3822 - 0.03133\,DA(\%) \quad (4)$$

The results obtained using both equations are reported in Table 4.

It can be appreciated that the method of Baxter gave much lower degrees of acetylation for the three samples, although both methods of *DA* determination by IR coincide in reporting a higher *DA* for the sample obtained by demineralizing with CO_2.

3.1.3 X-ray Diffraction Pattern

Figure 2 shows the X-ray diffraction patterns of lobster shell before and after deproteinization together with those of chitins isolated by the three demineralization procedures used here. The diffraction pattern of calcite is also shown for comparison.

The occurrence of $CaCO_3$ in the lobster shell is evidenced by the presence of the peaks of calcite in its diffraction pattern. After deproteinization the contribution of the calcite peaks to the diffraction pattern of the material becomes more prominent. On the other hand, chitins prepared by the three procedures almost exclusively exhibited the typical crystalline peaks of α-chitin, particularly the major ones at 2θ = 19 and 9° [22]. No significant difference can be seen in the diffraction patterns of CHI-HCl and CHI-CO_2. However, in the case of the chitin sample prepared by the treatment with EDTA, some contribution of the main calcite diffraction peak at 2θ = 29.4° can be perceived.

X-ray diffraction patterns allowed calculating the crystallinity index (CrI) of the chitins obtained by the three procedures. To this end we followed the method used by Osorio-Madrazo *et al*. [23] for the determination of CrI of chitosan. Briefly, the values of CrI are calculated from the separated crystalline peak areas of the diffractogram. We first estimate the diffractogram of the amorphous contribution directly on the sample diffractogram, as indicated in Figure 3 for CHI-HCl. Then, the crystalline contribution area (A_{crist}) between

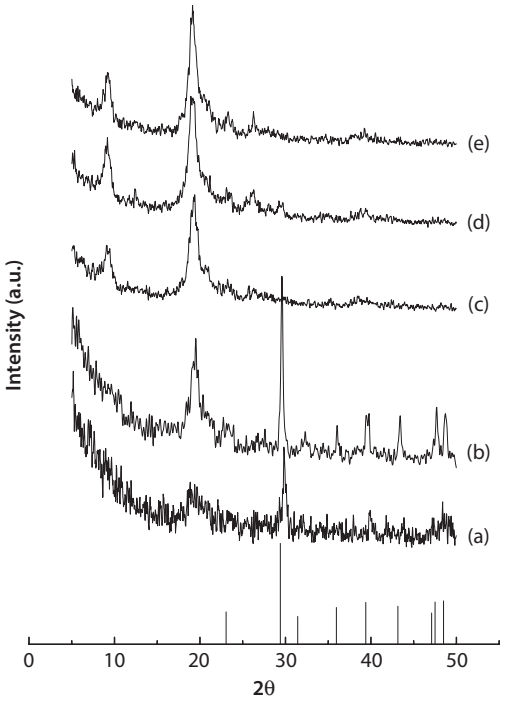

Figure 2 Powder X-ray diffraction patterns of lobster shell and chitins obtained by different procedures: (**a**) Lobster shell; (**b**) deproteinized shell; (**c**) CHI-HCl; (**d**) CHI-EDTA; (**e**) CHI-CO_2. The lines at the bottom correspond to the peaks reported for calcite [26], represented in the graph with their relative intensities.

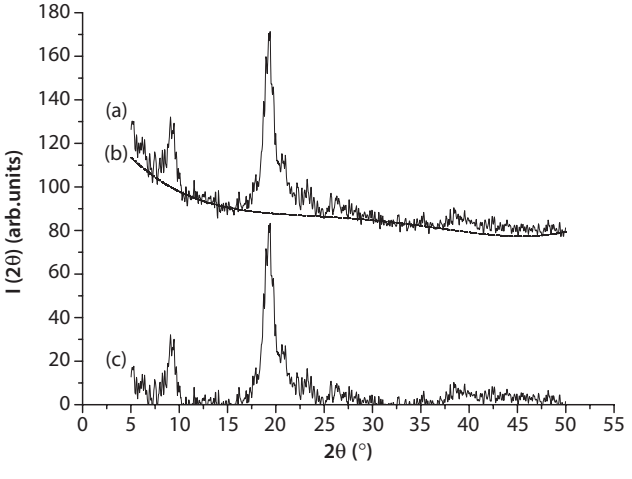

Figure 3 Procedure for the calculation of crystallinity indexes from X-ray diffraction pattern of chitin prepared by demineralization with HCl (CHI-HCl). (**a**) Diffraction diagram of the original chitin CHI-HCl. (**b**) Estimated cubic spline curve for the amorphous contribution of CHI-HCl in the X-ray diffraction pattern. (**c**) Assessed crystalline contribution of CHI-HCl after subtracting the amorphous estimate (**b**) from the total diffraction pattern (**a**).

7 and 50° (2θ) is obtained from the subtraction of this amorphous contribution to the total diffractogram of CHI-HCl (A_{total}). This permitted us to calculate $CrI = A_{crist}/A_{total}$ by integration of the crystalline peaks and the total diffraction pattern between 7 and 50° (2θ) (Figure 3). The resulting CrI of chitins were 33% for CHI-HCl, 49% for CHI-EDTA and 53% for CHI-CO_2. In spite of the fact that this calculation of the CrI might overestimate to some degree the contribution of the amorphous phase [23], the results indicate that demineralization with CO_2 gives the most crystalline chitins.

3.1.4 *Thermal Characterization*

The DTA, TG and DTG curves of the spiny lobster shell before and after the deproteinization step are shown in Figure 4(a). The first endothermic peak of lobster shell at 100 °C with a weight loss of 7.7% corresponds to the elimination of water. Further decomposition of the sample occurred between 190 and 780 °C. In the DTG curve of lobster shell, peaks are observed at 198, 335, 477, 552 and 714 °C. The first four decomposition processes are exothermic, as shown by the DTA curve, and are related to the degradation of the organic compounds of the shell (proteins, chitin, pigments and lipids), while the peak at 714 °C is endothermic and is due to the decomposition of the inorganic material. These results are in qualitative agreement with those reported by García [24], although in that work the DTA curves were obtained in air.

After deproteinization the material consists mainly of chitin and inorganic components, and the DTA curve below 600 °C of deproteinized shell in Figure 4(a) resembles the DTA pattern of chitin samples depicted in Figure 4(b). In this figure the DTA, TG and DTG profiles of chitin samples obtained by the three demineralization procedures used in the present work are shown. It becomes clear that the thermal behavior of chitins depends on the demineralization procedure employed. The samples obtained by the HCl treatment started to decompose at lower temperatures. The first exothermic peak for CHI-HCl decomposition has a maximum at 364 °C, while for CHI-EDTA and CHI-CO_2 it is at 390 °C. The maximum in the DTG curves observed at 332, 348 and 360 °C for CHI-HCl, CHI-EDTA and CHI-CO_2, respectively, also points to the lower thermal stability of the sample demineralized with HCl. At the same time, it indicates that the more thermally stable chitin was the one obtained using CO_2.

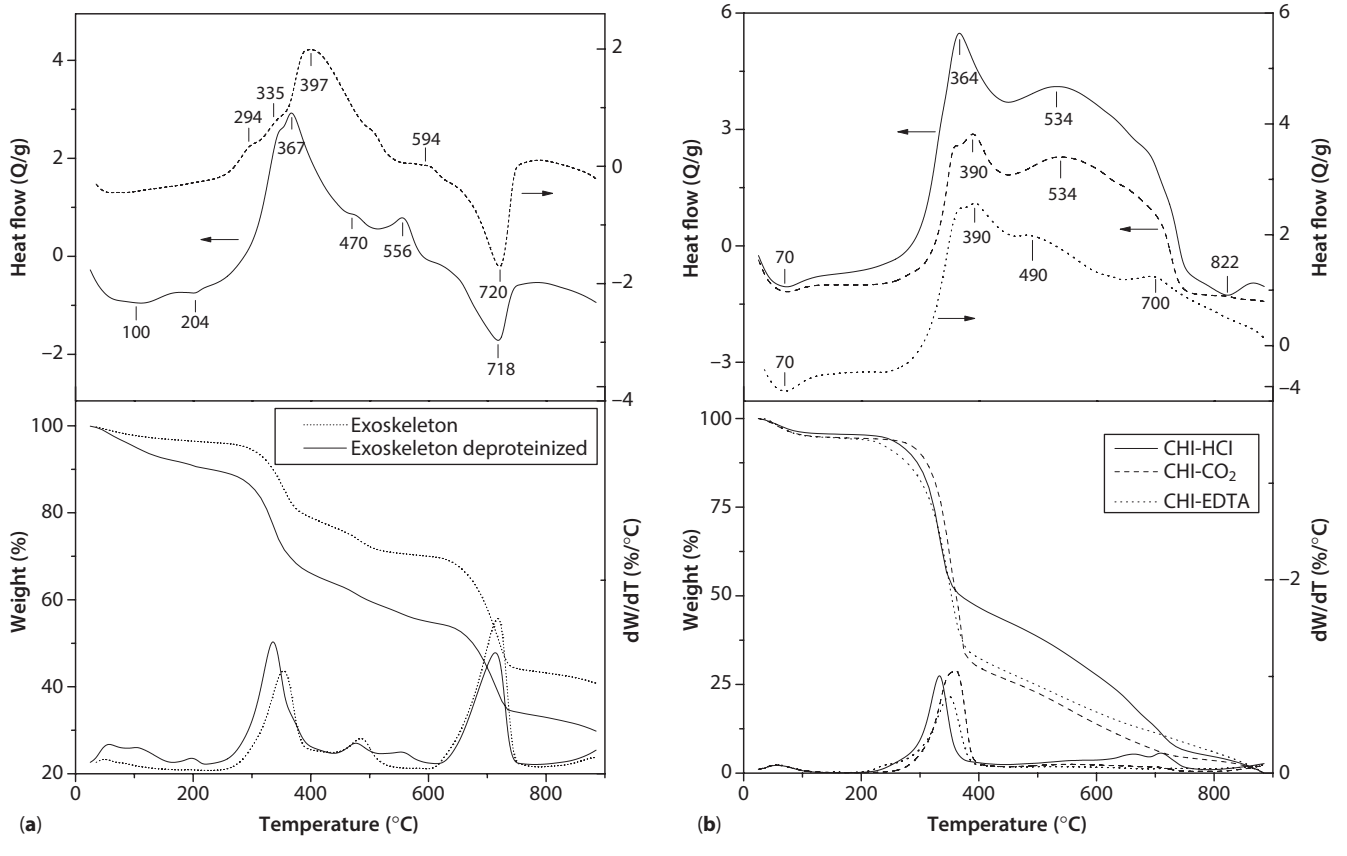

Figure 4 Thermoanalytical curves of (**a**) lobster shell and (**b**) chitins obtained by different procedures.

Thermal analysis can be used to evaluate the degree of acetylation of chitin [25]. It has been shown that the weight loss (X) associated with the main thermal decomposition effect of chitin is related to the percentage of acetyl groups (Y) of the sample as follows:

$$Y = 0.28X - 0.23 \quad (5)$$

Before evaluating Y for the three chitins prepared, the weight losses have to be referred to as moisture and ash free sample [25]. Having estimated Y, the degree of acetylation, DA, can be readily obtained by the relationship $DA = 100Y/21.1$, where 21.1 corresponds to the percentage of acetyl groups of a fully acetylated chitin. The results are listed in Table 4. In spite of the difference obtained in the absolute values of *DA* obtained with those of IR spectroscopy, again it turns out that the higher acetylation degree was that of the sample obtained by demineralizing with CO_2.

4 CONCLUSIONS

Deproteinized lobster shells were demineralized using CO_2 and a cationic exchange resin. With this procedure, the ash content of the material was lowered from 67 to 1.3%. The use of the cationite is essential, because with CO_2 alone the ash content was only reduced to 20%. Chitin obtained by this method was characterized and compared with chitins obtained by demineralization with HCl and with EDTA. Similar ash contents were obtained in the three cases, but chitin obtained using CO_2 and a cationite exhibited greater degree of acetylation, higher crystallinity and greater thermal stability than the ones obtained with HCl or EDTA. These better properties of CHI-CO_2 are a consequence of the milder demineralization conditions of this process. Therefore, the use of CO_2 and a cationite for demineralizing lobster shells is a promising alternative to the conventional HCl and EDTA treatments for obtaining chitin.

REFERENCES

1. M.G. Peter, Chitin and chitosan in fungi, in: *Polysaccharides II: Polysaccharides from Eukaryotes*, A. Steinbüchel (Ed.), pp. 123–157, Wiley-VCH, Weinheim (2002).
2. G.A.F. Roberts, *Chitin Chemistry*, pp. 54–61, The Macmillan Press Ltd., London (1992).
3. H.K. No and P. Meyers, Preparation of chitin and chitosan, in: *Chitin Handbook*, R.A.A. Muzarelli and M.G. Peter (Eds.), pp. 475–489, European Chitin Society, Atec, Grottammare (1997).
4. R.A.A. Muzzarelli. *Chitin*, pp. 87–94, Pergamon Press, Oxford (1977).
5. J. Synowiecki, The recovery of protein hydrolyzate during enzymatic isolation of chitin from shrimp Crangon processing discard. *Food Chem.* **68**, 147–152 (2000).
6. C.J. Brine and R. Austin, Chitin variability related with species and methods of preparation. *Comp. Biochem. Physiol.* **69**, 283–286 (1981).
7. Y. Qin, X.M. Lu, N. Sun, and R.D. Rogers, Dissolution or extraction of crustacean shells using ionic liquids to obtain high molecular weight purified chitin and direct production of chitin films and fibers. *Green Chem.* **12**, 968–971 (2010).
8. M.A. Ramírez, L. Alfonso, P. González, J.R. Fagundo, M. Suarez, C. Melián, T. Rodríguez, and C. Peniche, Kinetics of the demineralization reaction of deproteinized lobster shells using CO_2. *J. Renew. Mater.* **3**, 73–80 (2015).
9. J. Fajón and J.R. Fagundo, Geochemisty of waters in tropical karst mountains in western Cuba, in: *Management of Water Resoures in Protected Areas*, H. Farfán, J.L. Corvea, I. Gutiérrez, and J.W. LaMoreaux (Eds.), pp. 221–232, Springer, New York, NY (2013).
10. Standard Methods for the Examination of Water and Wastewater, APHA, AWWA, WPCF (1992).
11. S.S.M. Sun, MicroLowry method, in: *Methods in Plant Molecular Biology and Agricultural Biotechnology: A Laboratory Training Manual*, pp. 9–11, Asian Vegetable Research and Development Center, Taipei (1994).
12. A. Chandumpai, N. Singhpibulporn, D. Faroongsarng, and P. Sornprasit, Preparation and physico-chemical characterization of chitin and chitosan from the pens of the squid species. *Loligo lessoniana* and *Loligo formosana*. *Carbohydr. Polym.* **58**, 467–474 (2004).
13. F. Boßelmann, P. Romano, H. Fabritius, D. Raabe, and M. Epple, The composition of the exoskeleton of two crustacea: The American lobster *Homarus americanus* and the edible crab. *Cancer pagurus*. *Thermochim. Acta* **463**, 65–68 (2007).
14. M. Rinaudo, Chitin and chitosan: Properties and applications. *Prog. Polym. Sci.* **31**, 603–632 (2006).
15. M.A. Ramírez, A.T. Rodríguez, J.A. Alfonso, J.A. Azocar, Y. Vázquez, L. Alfonso, and C. Peniche, Composición química y elementos trazas en subproductos de exoesqueletos de langosta Panulirus argus con posible uso agrícola. *Rev. CENIC Quím.* **41**, 99–104 (2010).
16. S. Hajji, I. Younes, O. Ghorbel-Bellaaj, R. Hajji, M. Rinaudo, M. Nasri, and K. Jellouli, Structural differences between chitin and chitosan extracted from three different marine sources. *Int. J. Biol. Macromol.* **65**, 298–306 (2014).
17. J. Jung and Y. Zhao, Alkali- or acid-induced changes in structure, moisture absorption ability and deacetylating reaction of β-chitin extracted from jumbo squid (*Dosidicus gigas*) pens. *Food Chem.* **152**, 355–362 (2014).
18. T. Nidheesh and P. Suresh, Optimization of conditions for isolation of high quality chitin from shrimp processing raw byproducts using response surface methodology and its characterization. *J. Food Sci. Technol.* **52**, 3812–3823 (2015).
19. W. Argüelles-Monal and C. Peniche-Covas, Study of interpolyelectrolyte reaction between chitosan and carboxymethyl cellulose. *Makromol. Chem. Rapid Commun.* **9**, 693–697 (1988).

20. A. Baxter, M.l. Dillon, K.D.A. Taylor, and G.A.F. Roberts, Improved method for i.r. determination of the degree of N-acetylation of chitosan. *Int. J. Biol. Macromol.* **14**, 166–169 (1992).
21. J. Brugnerotto, J. Lizardi, F.M. Goycoolea, W. Argüelles-Monal, J. Desbriéres, and M. Rinaudo, An infrared investigation in relation with chitin and chitosan characterization. *Polymer* **42**, 3569–3580 (2001).
22. F.M. Goycoolea, W. Argüelles-Monal, C. Peniche, and I. Higuera-Ciapara, Chitin and chitosan, in: *Novel Macromolecules in Food Systems*, G. Doxastakis and V. Kisseoglou (Eds.), pp. 265–307, Elsevier, Amsterdam (2000).
23. A. Osorio-Madrazo, L. David, S. Trombotto, J.-M. Lucas, C. Peniche, and A. Domard, Kinetics of the solid-state acid hydrolysis of chitosan: Evolution of the crystallinity and macromolecular structure. *Biomacromolecules*. **11**, 1376–1386 (2010).
24. I. García, Thermoanalytical studies on lobster shell. *J. Therm. Anal*. **27**, 257–262 (1983).
25. I. García, C. Peniche, and J.M. Nieto, Determination of the degree of acetylation of chitin and chitosan by thermal analysis. *J. Therm. Anal.* **28**, 189–193 (1983).
26. D.L. Graf, Crystallographic tables for the rhombohedral carbonates. *Am. Mineral*. **46**, 1283–1316 (1961).