Chitin Characterization by SEM, FTIR, XRD, and ^{13}C Cross Polarization/Mass Angle Spinning NMR

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ABSTRACT: The full characterization of chitin obtained from squid, shrimp, prawn, lobsters, and king crab is reported. Elemental analysis, including metals such as Ca, Mg, Zn, Cd, Hg, Cr, Mn, Cu, and Pb, was performed, which is quite relevant because the skeleton composition is slightly different for each species. The morphology was studied by means of TEM and their compositions were determined by energy-dispersive X-ray analysis. ^{13}C cross polarization/magic angle spinning NMR was applied to determine the chemical shift of all the carbons and the difference between them. Chitin was isolated by using chemical methods, alternating hydrochloric acid and sodium hydroxide. The α-chitin from shrimp, prawn, lobsters, and king crabs showed two signals at 73.7 and 75.6 ppm. Meanwhile, the β-chitin from squid exhibited one signal at 75.2 ppm. FTIR studies were used to analyze α-chitin from shrimp and β-chitin from squid. The α-chitin exhibited amide I vibration modes at 1660 and 1627 cm^{-1}, whereas the β-chitin showed one band at 1656 cm^{-1}. X-ray diffraction showed that α-chitin is orthorhombic (a = 4.74 Å, b = 18.86 Å, and c = 10.32 Å) and β-chitin had a monoclinic dihydrated form (a = 4.80 Å, b = 10.40 Å, c = 11.10 Å, and β = 97°). J Appl Polym Sci 93: 1876–1885, 2004

Key words: α- and β-chitin; ^{13}C CP/MAS NMR; FTIR; morphology; polysaccharides

INTRODUCTION

Chitin is the second most abundant polysaccharide in nature after cellulose. It can be found in the arthropodes exoskeleton, fungus walls, and in some algae.[1] However, for its extraction for commercial purposes, the crustacean shells from waste fisheries are used. It is also a material that protects the exoskeleton of insects, shielding them from harm and pressure. It is broken down by bacteria in the ocean through enzymatic degradation.

Chitin, which enables attachment of a variety of organic contaminants, minerals, and oils, is a material that can be used in the environmental clean up of ocean oil spills. Its efficiency in filtering systems improves with its use as a natural coagulant that traps dirt and debris. Currently, it is used as a cleaning medium for the water treatment of pools. The overall water quality is cleaner, and is less staining compared with pool water not treated with chitin.

In Chile, in 2001, 1754 tons/year of red shrimp *Pleuroncodes monodon* (Ultzer) were caught.[2] As a consequence, the fisheries produce a crustacean waste of approximately 526 tons. According to the United Nations Food and Agricultural Organization (FAO) the global production of shrimp is about three times that of crab from 1995 to 1999 (crab and shrimp at 1,299,464 and 4,021,521 metric tons, respectively).

In this work the shrimp skeleton as a source of chitin was studied.

EXPERIMENTAL

Materials

Skeletons from shrimp, prawn, king crabs, crabs, lobster, and squid were used.

Methods

Chitin

The chitin was obtained by normal treatment with HCl and NaOH 2*M* at constant stirring for 2 h each.

Analysis of red shrimp

Humidity was determined for the red shrimp shell (*Pleuroncodes monodon*) and for chitin from several other sources. Each sample was placed in a porcelain crucible and heated at 105°C in an oven to constant

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TABLE I
Chemical Characterization from Red Shrimp Shell (*Pleuroncodes monodon*)

	Parameter							
	Humidity (w/w) (%)	Chitin (w/w) (%)	Protein (w/w) (%)	Ash (w/w) (%)	C (w/w) (%)	N (w/w) (%)	Ca (g/kg)	Mg (g/kg)
Value	9.11	17.00	21.90	42.50	47.62	3.54	149 ± 3	12.4 ± 0.5

weight. The dried sample was placed in an oven and heated at 900°C to constant weight to determine the ash content.[3]

Ashes were dissolved in nitric acid to determine the metal content by atomic absorption. A Unicam series spectrophotometer (Spectronic Unicam, Cambridge, UK) with cathodic lamp was used.

The percentage chitin present in the shell was determined by the method of Black and Schwartz [4] and the protein content was determined from N (percentage) × 6.25.

FTIR spectroscopy

The infrared spectra were registered in an FTIR Nicolet Magna (Nicolet Analytical Instruments, Madison, WI) connected to a PC with Omnic software (Thermo Electron Corp., Woburn, MA) for data processing. The samples were prepared in KBr pellets at a concentration of 2% (w/w). The spectra were obtained with 4 cm^{-1} resolution and 64 scans.

NMR spectroscopy

The ^{1}H-NMR and ^{13}C-NMR were obtained at 353 K in a Bruker AC 300 spectrometer (Bruker Instruments, Darmstadt, Germany). The purified samples were dissolved at a concentration of 10 mg/mL in deuterated water (99.9%) for ^{1}H and 70 mg/mL for ^{13}C. A drop of concentrated HCl or NaOH, to dissolve the samples, was added. Some samples were lyophilized after being dissolved and dissolved again in the same solvent to avoid excess humidity. 3-Trimethylsilylpropane sodium sulfonate (DSS) and tetramethylsilane (TMS) were used as references for ^{1}H- and ^{13}C-NMR. The solid-state ^{13}C CP/MAS NMR spectra were registered in a Bruker AMX 300. In all cases 3072 scans were accumulated. The contact time was 1 ms, the repetition time 5 s, and the acquisition time 50 ms. DSS was used as an internal reference (0 ppm).

TEM analysis

Parts of the cephalothorax from red shrimp were selected then fixed in a resin, and the samples were cut transversally with an ultra microtome. The slices were placed in a Cu grid of 100 mesh and the specimens were observed in a JEOL JEM 1200 EX II TEM equipment (JEOL, Tokyo, Japan).

The spectroscopic studies of energy dispersion were carried out in a transmission electron microscope using energy-dispersive X-ray (EDX) with a Noran Model 611K-3555 detector (Altran Corp., Boston, MA). The spectra, with characteristic signals for each element using the K_α energy level, were obtained.

SEM analysis

The samples were fixed in a sample holder and covered with a gold layer for 3 min using an Edwards S150 sputter coater (BOC Edwards, São Paulo, Brazil). Then the samples were placed in an ETEC autoscan Model U-1 scanning electron microscope (The University of Massachusetts, Worcester, MA).

X-ray analysis

The X-ray powder diffractograms of the polymers were obtained in a Siemens D-5000 diffractometer (The University of Reading, UK) with radiation Cu–K_α (40 kV and 30 mA) provided with a graphite monochromator ($\lambda = 1.5418$ Å) at 298 K. The relative intensity was registered in a dispersion range (2θ) of 4–40°.

RESULTS AND DISCUSSION

To determine the chemical composition of a shell, a fresh sample was used, dried, and crushed, and the main components were determined. The obtained results are shown in Table I.

All the present determinations were obtained on a dry basis after determining the humidity percentage. The metal content was determined from the sample after calcination at 900°C. In Table I it can be observed that the red shrimp shells exhibit the highest value of ash, 42.5% (w/w). These salts are mainly $CaCO_3$ and $MgCO_3$.[5] The chitin content is around 17% (w/w); proteins, 21.9% (w/w); humidity, 9.11% (w/w); and 10.5% are pigments and lipids.[1] The results of Table I are in agreement with the composition found in other crustacean shells.[6]

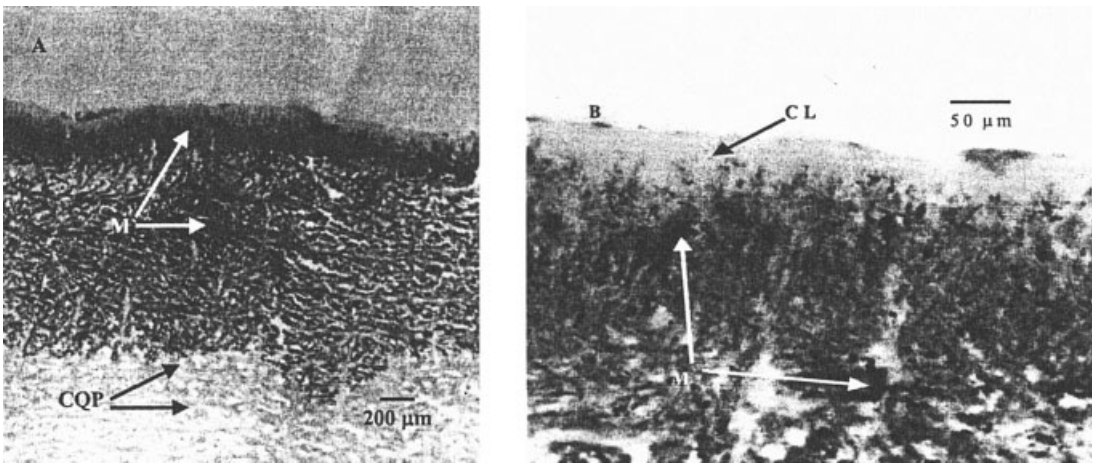

Figure 1 Transmission electron micrograph obtained from a transversal cut of cephalothorax from red shrimp. CL, lipidic layer; CQP, chitin protein complex; M, metals.

Morphological studies of red shrimp exoskeleton

To study the morphology and the components in the red shrimp shells a transversal slice of the cephalothorax was prepared and observed by TEM (Fig. 1). The cephalothorax was chosen because it represents the main source of product waste. The thickness of the cephalothorax is about 1 mm.

In the micrograph of Figure 1(A), four different zones are observed in the cephalothorax. The analysis, from the top surface to the bottom, shows a thin transparent layer with thickness around 62 nm [Fig. 1(B)]. This corresponds to the lipidic layer present in all the crustaceans.[1] In such a layer it has been suggested that all the pigments are solubilized because of the similar polarities between them.[1] Then a dark region (I) appears, next to the upper layer, and another less dark region (II) limiting with the clear region (III). In regions II and III [Fig. 1(A)] one can observe organized structures corresponding to the chitin–protein complex, which have been described for other arthropods.[1]

Figure 1(B) shows a magnification of region I and it can be observed that it is formed by the same component as region II. Through use of EDX analysis it is possible to analyze the elements present in two different regions of the shell (II and III). The results obtained are shown in Figure 2.

The Cu and Cr present in EDX are attributed to the copper grid used as support. The EDX result from region II appears in Figure 2 B and shows the existence

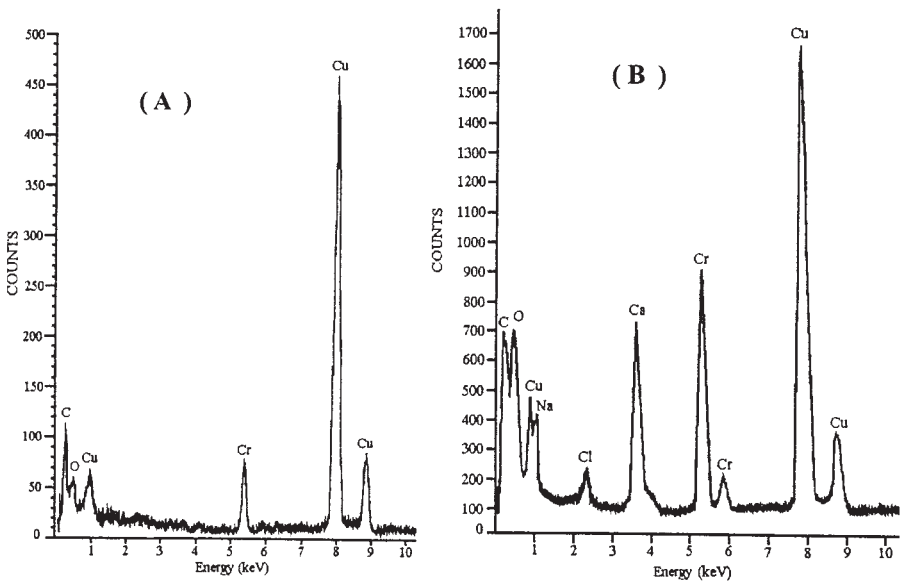

Figure 2 TEM–EDX analysis carried out on a transverse slice of red shrimp (*Pleuroncodes monodon*): (A) region III; (B) region II.

TABLE II
Metal Composition of Red Shrimp Shell

Metal	Concentration (mg/kg)
Fe	708 ± 2
Pb	<1.6
Cr	5.92 ± 0.10
Cd	2.03 ± 0.03
Zn	38.3 ± 0.5
Mn	34.5 ± 0.2
Cu	22.2 ± 0.2

of metals (Ca and Na). No metals were detected in region III [Fig. 2(A)]. The presence of NaCl in the sample is attributed to the sea water, given that no previous treatment of the sample was carried out. On the other hand, the C and O come from the organic compounds present in all the shells from the chitin and proteins and some inorganic salt like $CaCO_3$. Figure 2(B) shows the signals corresponding to Ca, C, and O that are similar to and more intense than that of NaCl.

Besides, the signals in region II for C and O are much greater than those in region III. This suggests that the presence of organic compounds is exclusively in region II. Table II summarizes the other metals present in the shells attributed to sea water contamination.

The presence of Ca in the shell agrees with the elemental analysis previously carried out (see Table I). In these studies by TEM (Fig. 1) it is interesting to observe that metals are located in the surface layer of approximately 212 nm inside the exoskeleton, which is also more dense (region I). This is explained by the fact that the surface must support mechanical stress. It is known that Ca and other metals act as nuclei to produce crosslinking between α-chitin and proteins.[1] The process in which metals are used to form a natural composite is known as sclerotization. Because of the crustacean scleroticides, this can be used as protection against environmental changes (temperature and pressure) and against the attack of natural predators.[7]

Chitin and chitosan characterization

The most important properties of chitin and chitosan are the molecular weight and the degree of acetylation (DA), which depend on the raw materials and the method used.[6] For that reason, it is necessary to determine more precisely both parameters in each sample.

Because of the several methods and material sources used to obtain chitin it was necessary to design a process according to our conditions. In our laboratory, methodology was adapted to obtain chitin from the exoskeleton of red shrimp, *Pleuroncodes monodon*.[8] Such a process deals with depigmentation with acetone using a liquid to solid ratio of 1 : 10 (w/v). This treatment can be avoided when the raw material is dried under the sun (more than 1 week) and the amount of pigment decreases substantially. According to the literature the demineralization and deproteinization processes can be done in any order and a number of times as necessary. In our laboratory an experimental process was designed where several variables were considered such as chitin and ash contents.[8] The best results were obtained when 2*N* HCl was used with a solid–liquid relation of 1 : 15 (w/v) at room temperature for 1 h.

After deproteinization with 4% NaOH in a solid–liquid relation 1 : 20 (w/v) at 100°C for 3 h, products were obtained with less than 1% of ash and more than 89% of chitin. The chitin can be used for chitosan production because remnant proteins and pigments can be removed in the chitosan process.

In this work chitins from different sources were also characterized and the susceptibility to lisozyme hydrolysis was compared in relation to their sources. It is important to notice that chitins from shrimp, prawn, and king crabs were obtained in our laboratory by different procedures, whereas the lobster and squid chitins were not obtained in our laboratory. The results of chemical characterization of these biopolymers are shown in Table II.

From Table III we can see that the humidity content in the α-chitins is generally less than 7% but, in the squid chitin, it is near 14%. In all cases the ash content is lower than 1%, which is indicative that demineralization is quite efficient. The percentage of nitrogen is corrected according to ash and humidity for each sample. The experimental values of N% are near the theoretical (6.89) calculated for a chitin that is completely deacetylated. The nitrogen content is also a measure of the protein amount still present in the chitin. A low experimental value of N is indicative of the minimum amount of protein left. The sample composition and purity of polysaccharides are in the range of commercial chitins. In all the samples the degree of acetylation (DA) is quite homogeneous. The reduced viscosity

TABLE III
Chemical Characterization of Chitins

Sample	N (%)	DA (%)	η^{a}_{red}	Humidity (%)	Ash (%)
King crab chitin	6.8	79.5	301.6	3.8	0.59
Shrimps chitin	6.8	78.7	2262.3	5.9	0.49
Crab chitin	6.6	83.9	174.7	5.2	0.32
Squid chitin	6.7	95.1	2175.7	13.6	0.27
Lobster chitin	6.8	84.6	150.3	6.9	0.50

[a] It was obtained with the solvent *N*,*N*-dimethylacetamide/LiCl 5% (w/v) at a polymer concentration of 0.03 g/dL at 30°C.

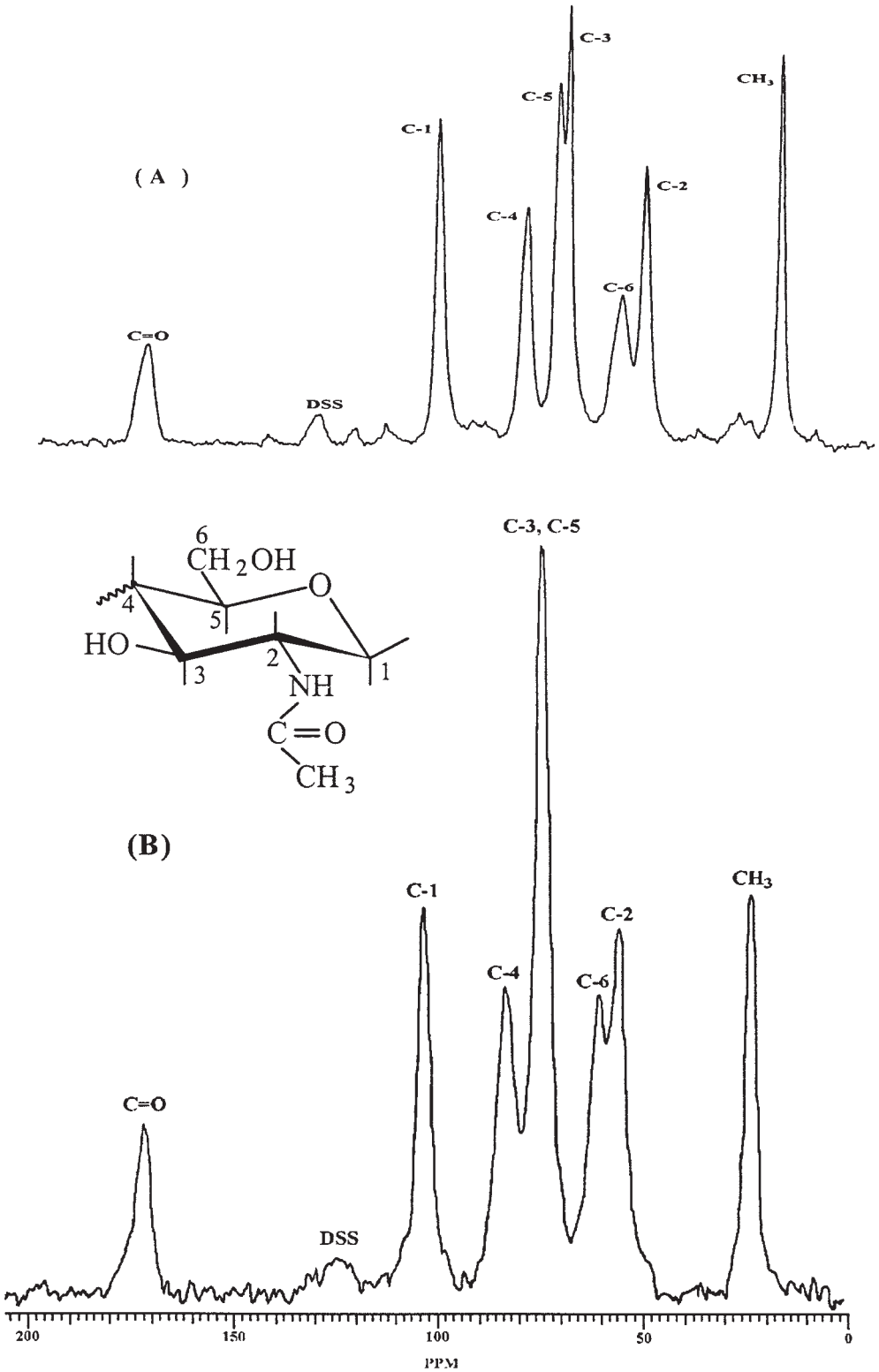

Figure 3 ^{13}C CP/MAS NMR spectra from shrimp chitin (A) and squid chitin (B).

(η_{red}), related directly with the M_v, changes from one sample to another. However, the highest M_v corresponds to chitin from shrimp. It is interesting that β-chitin from squid has the greatest acetyl content (95%) and molecular size similar to the shrimp chitin. This result may be explained by the less severe process used and because of the raw material that is obtained in a purer form.[8]

TABLE IV
Chemical Shift (ppm) in ^{13}C CP/MAS NMR for α- and β-Chitins

Sample	Carbon							
	C1	C2	C3	C4	C5	C6	C═O	$-CH_3$
Shrimp chitin	104.4	55.7	73.7	83.6	75.6	61.5	173.7	23.2
Lobster chitin	103.0	54.2	72.7	82.1	74.5	59.5	172.3	21.8
Crab chitin	102.9	54.3	72.6	82.2	74.2	59.7	172.3	21.8
King crab chitin	103.1	54.5	72.7	82.2	74.4	60.1	172.5	21.9
Squid chitin	104.7	56.3	75.2	84.4	75.2	61.4	174.5	23.8

NMR analysis

In Figure 3 the ^{13}C CP/MAS NMR of α- and β-chitin are shown. The signals were assigned according to Tanner et al.[9] and are summarized in Table IV.

As we can see in Figure 3, the spectra of α-chitins [Fig. 3(A)] are quite different from those of β-chitin [Fig. 3(B)]. In the β-chitin from squid a signal appears at 75.2 ppm assigned to C3 and C5, whereas in the α-chitins from shrimp, the C3 and C5 appear as two signals characteristic of a partially solved doublet, at 73.7 and 75.6 ppm, respectively. This is attributed to the different configurations of C3 and C5 resulting from the hydrogen bonds formed. In the solid state the two types of acetamide groups (α-chitins) present are different in their hydrogen bonds and are responsible for the signal resolution.[9]

The DA determination by this technique uses the signals of carbonyl and methyl carbon atoms. However, Rinaudo et al.[10] demonstrated that we must be very careful to evaluate the DA from the ^{13}C solid-state NMR because the kinetics of the polarization process is not the same for each group. They found out that, whereas the magnetization of the carbon from the $-CH_3$ group and the glucopyranosic ring reaches 88% for the contact time usually used (1 ms), the carbonyl reaches only 84% of the theoretical value expected for this group. According to this argument, if the carbonyl signal is considered, a systematic error of 4% is achieved in the measurement.

Following this argument, in this work the relation between the intensities of the signal from the CH_3 group and all the intensities of all the carbons of the glucopyranosic ring were used to determine the DA, according to the following expression:

$$DA(\%) = \left(\frac{I_{CH_3}}{I_{C1} + I_{C2} + I_{C3} + I_{C4} + I_{C5} + I_{C6}/6} \right) \times 100 \quad (1)$$

The results of degree of acetylation obtained from the samples according to this relation are shown in Table III.

FTIR spectroscopy

The chitins are always characterized using FTIR (Fig. 4). Only the spectra of shrimp and squid are shown because all other spectra obtained are quite similar and correspond to α-chitins. In Table V the signals from both spectra are summarized.

The α- and β-chitins can be distinguished by FTIR spectroscopy because of the different hydrogen bonds. The more obvious spectral difference is the frequency of the vibration modes of amide I in the region 1660–1620 cm^{-1}. In the α-chitin two absorptions are observed at 1660 and 1627 cm^{-1} and in the β-chitin only one band at 1656 cm^{-1}.[11] Nowadays, the most accepted explanation is the existence of two types of amides.[9] Half of the carbonyl groups are bonded through hydrogen bonds to the amino group inside the same chain (C═O···H—N) that is responsible for the vibration mode at 1660 cm^{-1}. The rest produces the same bond plus another with the group $-CH_2OH$ from the side chain. This additional bond produces a decrease in the amide I band at 1627 cm^{-1}.[12] The existence of these interchain bonds is responsible for the high chemical stability of the α-chitin structure. In the β-chitin all the amide groups participate only in the first kind of hydrogen bonds and the amide I band appears at 1656 cm^{-1}, which is a 29 cm^{-1} shift from the second band of the α-chitin because the distance C═O···H—N is slightly longer.

In the corresponding region of the OH and NH (3600–3000 cm^{-1}) groups the α-chitin exhibits a more detailed structure than that of the β-chitin. This is attributed to the different packing arrangements of the macromolecules. The shoulder that appears in the α-chitin spectrum at 3479 cm^{-1} is attributed to the intramolecular hydrogen bond involving the OH(6)···O═C that is missing in the β-chitin. In the shrimp chitin a band appears at 3448 cm^{-1}, which corresponds to the intramolecular hydrogen bond O(3)H···O(5) from the ring.[13] This vibration appears at a lower frequency (3426 cm^{-1}) in the squid chitin. The bands at 3268 and 3106 cm^{-1} are assigned in the α-chitin to the vibrational modes of the NH of the amide (intermolecular hydrogen bond C═O···H—N and the NH groups intramolecularly bonded by H, respectively).

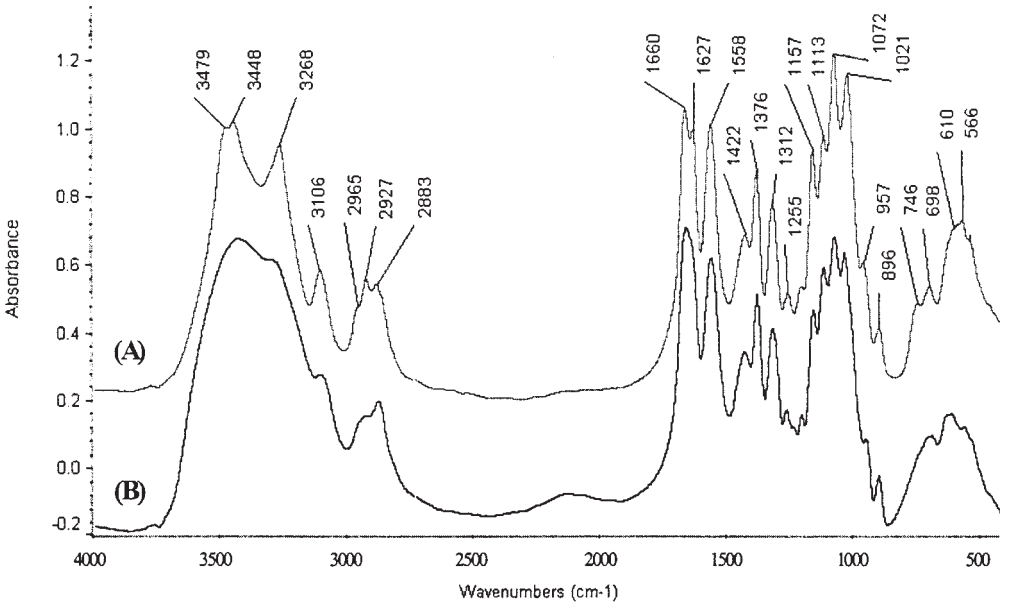

Figure 4 FTIR of α-chitin from shrimp (A) and β-chitin from squid (B).

Several authors postulate that a vibration-mode frequency, from the C—H bonds of the anomeric carbon atoms, can be used to characterize the configuration of the anomeric center from the glucopyranosicyclic residues: C—H axial at 891 ± 7 cm^{-1} and C—H equatorials at 844 ± 8 cm^{-1}.[14] The shrimp chitin absorbs at 896 cm^{-1} and the squid at 902 cm^{-1}, which is characteristic of the β-configuration in the anomeric center (C1) of this polysaccharide.

X-ray diffraction

To obtain the crystalline degree of the samples, an X-ray study was carried out. Figure 5 shows the dif-

TABLE V
FTIR of the Main Signals of Chitins

α-Chitin absorption (cm^{-1})	β-Chitin absorption (cm^{-1})	Assigned
3479,3448	3479, 3426	ν_{OH}
3268	3290	ν^{as}_{NH}
3106	3102	ν^{s}_{NH}
2965	2962	$\nu^{as}_{CH_3}$
2927	2929	$\nu^{s}_{CH_2}$
2883	2880	$\nu^{as}_{CH_3}$
1660,1627	1656	$\nu_{C=O}$ (Amide I)
1558	1556	ν_{C-N} (C—N—H) + δ_{NH} (Amide II)
1422	1424	δ_{CH_2}
1376	1376	δ_{CH} + δ_{C-CH_3}
1312	1314	ν_{C-N} + δ_{NH} (Amide III)
1255	1262	δ_{NH}
1157	1155	ν^{as}_{C-O-C} (ring)
1072	1069	ν_{C-O}
1113	1111	ν_{C-O}
1021	1032	ν_{C-O}
957	948	γ_{CH_3}
896	902	γ_{CH} (C1 axial) (β bond)
746	—	ρ_{CH_2}
698	692	γ_{NH} (Amide V)
610	616	γ_{C-O}
566	—	γ_{C-C}

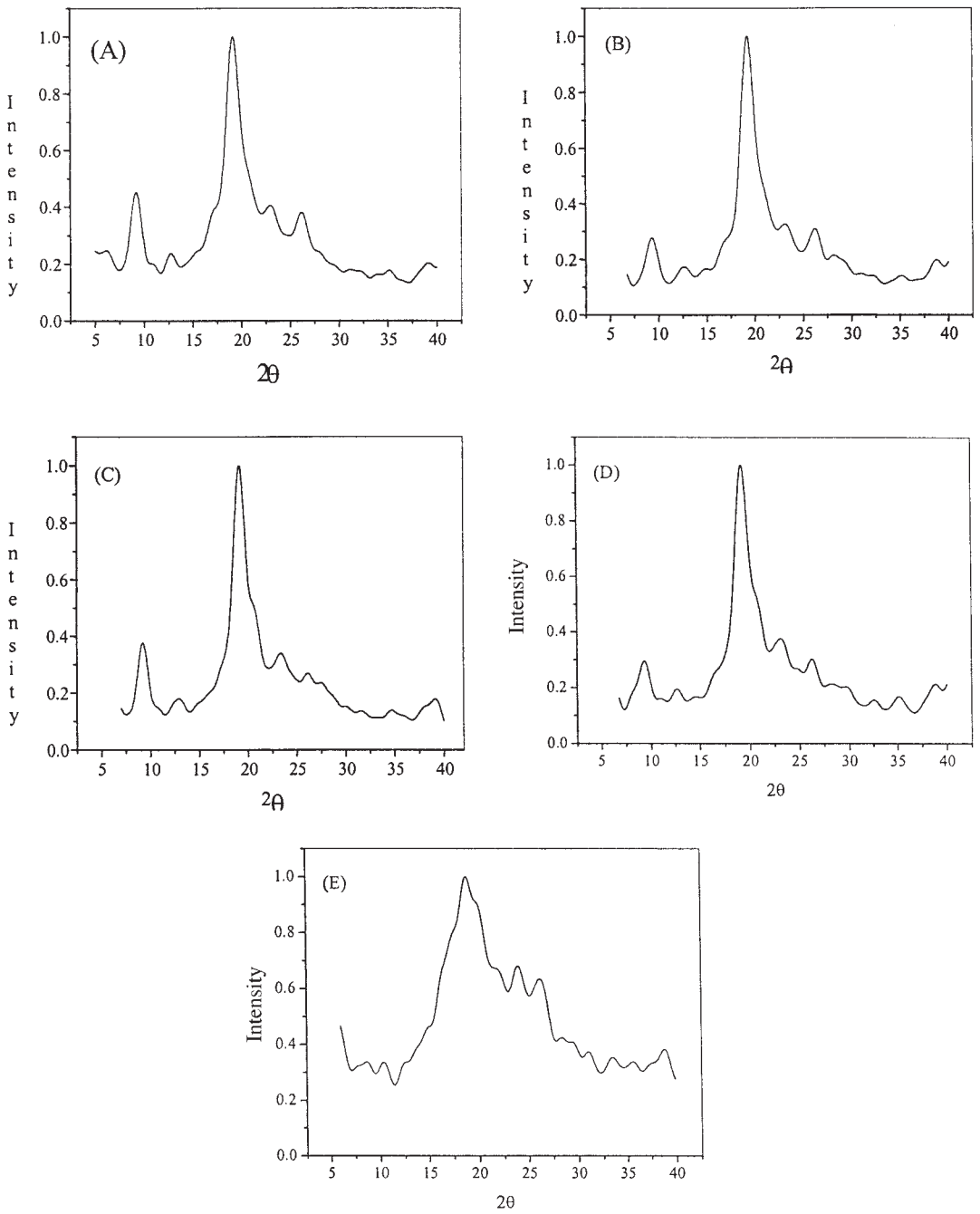

Figure 5 X-ray diffractograms of chitins from different sources: (A) shrimp; (B) lobster; (C) prawn; (D) king crab; (E) squid.

fractograms of different chitins. The crystalline index (CI) was determined according to the proposed method for cellulose and applied to these polymers[13] using the following expression[2]:

$$\text{CI}\ (\%) = [(I_{110} - I_{am})/I_{110}] \times 100 \qquad (2)$$

where I_{110} is the maximum intensity (arbitrary units) of the diffraction (110) at $2\theta = 19°$ and I_{am} is the intensity of the amorphous diffraction in the same unit at $2\theta = 12.6°$.

To calculate the *d*-spacing and the Miller index, values were used from the literature.[15] For α-chitin the dimension of the cell unit was considered with an orthorhombic structure ($a = 4.74$ Å, $b = 18.86$ Å, and $c = 10.32$ Å)[9] and for β-chitin monoclinic a dihydrated cell ($a = 4.80$Å, $b = 10.40$ Å, $c = 11.10$ Å, and $\beta = 97°$) was used, where β is the interaxial angle.[16] The results are shown in Table VI.

For an orthorhombic crystal the interplanar spacing (h, k, l) was determined by eq. (3) and for the monoclinic crystal by eq. (4), respectively:

$$d(h, k, l) = \left[\left(\frac{h}{a}\right)^2 + \left(\frac{k}{b}\right)^2 + \left(\frac{l}{c}\right)^2\right]^{-(1/2)} \qquad (3)$$

TABLE VI
Structural Parameters of Chitins Obtained from X-ray Diffraction

Sample	Plane	2θ	d (Å)	CI (RX)
Shrimp chitin (α)	(020)	9.22	9.65	
	(101)	12.76	4.63	
	(040), (110)	19.18	4.43	76.2
	(130)	22.98	3.85	
	(013)	26.18	3.39	
Lobster chitin (α)	(020)	9.32	9.65	
	(101)	12.62	4.63	
	(040), (110)	19.26	4.43	82.7
	(130)	23.16	3.85	
	(013)	26.22	3.39	
Crab chitin (α)	(020)	9.24	9.65	
	(101)	12.90	4.63	
	(040), (110)	19.18	4.43	81.9
	(130)	23.36	3.85	
	(013)	26.14	3.39	
King crab chitin (α)	(020)	9.34	9.65	
	(101)	12.66	4.63	
	(040), (110)	19.20	4.43	80.6
	(130)	23.12	3.85	
	(013)	26.28	3.39	
Squid chitin (β)	(010)	8.64	8.43	
	(122)	10.42	7.35	
	(102)	12.55	5.63	66.3
	(110), (020)	18.78	4.48	
	(114)	23.94	3.56	
	(121)	26.24	3.19	

$$d(h, k, l) = \left[\frac{\left(\frac{h^2}{a^2}\right) + \left(\frac{l^2}{c^2}\right) - \left(\frac{2hl}{ac}\right)\cos\beta}{\sin^2\beta} \right]^{-(1/2)} \quad (4)$$

The crystalline degree obtained for the α-chitins from crustacea is greater than that of the β-chitin from squid. This is in agreement with their different crystalline forms. The shrimp chitin obtained in this work possesses the CI value.

SEM analysis

To better understand chitin morphology, some chitin flakes were isolated from different places of the exoskeleton and were observed by SEM. In Figure 6 the electron micrographs, obtained for chitin isolated from different parts of the exoskeleton, are shown.

It is observed that, independent of the part of the shrimp exoskeleton, the isolated chitin retains its fiber character. In both cases a rough surface without porosity is observed, which might be related to their high molecular packing, with inter- or intramolecular hydrogen bonds, imparting a high crystalline degree to α-chitin (see Table VI).

It is known that, in the deacetylation process used to obtain chitosan, the diffusion of concentrated alkaline inside the fiber is crucial and is inhibited by low porosity and a high crystalline degree of the sample.

CONCLUSIONS

By using several analytical techniques it is possible to establish differences in the structures and chemical compositions of chitin from different raw materials.

It is possible to differentiate, by spectroscopic and X-ray methods, α-chitin from β-chitin.

By FTIR it is also possible to obtain different absorption bands from α-chitin and β-chitin.

The ^{13}C CP/MAS solid NMR allows one to differentiate the chitins of the five species under study. There are small differences in chemical shifts between each carbon for different species.

The X-ray diffractograms also exhibit differences in intensity and distances of different planes.

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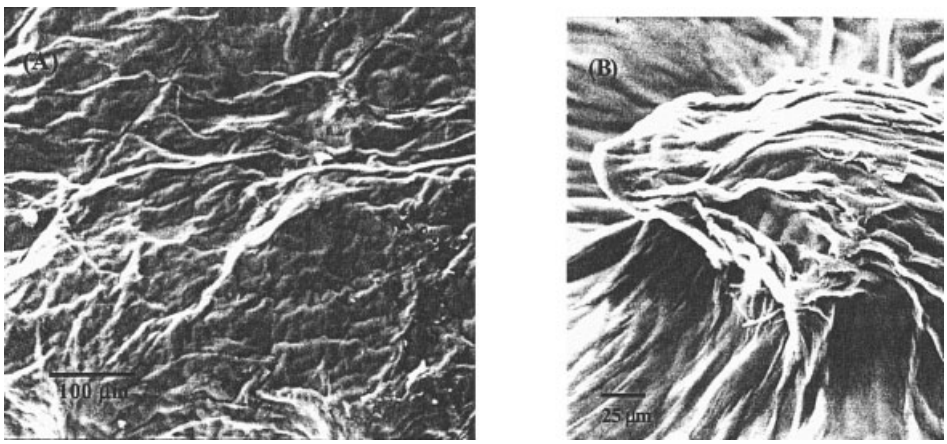

Figure 6 Scanning electron microscopy obtained from different parts of the exoskeleton.

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