CHARACTERIZATION OF CHITOSAN EXTRACTED FROM DIFFERENT ROMANIAN *BLACK* SEA CRUSTACEANS

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Chitosan from different types of crabs collected from the Romanian Black Sea Coast was extracted and characterized. The obtained chitosan powders were analysed and compared in terms of ash content, hygroscopicity and infrared spectroscopy. The purpose of this study is to evaluate which species are more suitable to extract a chitosan with good qualities. The obtained results showed that the marine source influences the chitosan properties. The extracted chitosan can find various uses in different industrial applications.

Keywords: chitin, chitosan, crabs, crustaceans, *Black* Sea

1. Introduction

After cellulose, the second most abundant natural polymer on Earth and the most abundant polymer in the marine environment is chitin [1], which represents the most important organic skeletal component of invertebrates and acts as a supporting material in many aquatic organisms like shrimp shell, krill, crabs, lobsters, prawn, mollusks, terrestrial organisms like scorpions or insects cuticle and in some microorganisms, mushrooms, cell walls of certain fungi and green algae [2-7].

The chemical structure of chitin consists of acetylated units of N-acetyl-D-glucosamine [1] (2-acetamido-2-deoxy-D-glucose), with β-(1→4) glycosidic bonds between each monomer [8]. The proportion of chitin in the crustacean shells may present variations with season and with species, but, generally, the exoscheletons contain 20-50 % calcium carbonate, 20-40 % protein, 15-40 % chitin and, additionally, other minor components such as pigments, lipids and metal salts [6, 8]. Chitin is a polysaccharide that has a very important derivative called chitosan [5, 9]. Chitosan is the deacetylated form of chitin and it is obtained

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by removing the acetyl groups from chitin as a result of a treatment with strong alkali solution (40-50 %) in specific conditions [3, 8], which are usually given by high temperatures (60-120 °C) for several hours [4, 10]. Chitosan chemical structure (fig. 1) is composed of randomly distributed β-(1→4)-linked D-glucosamine (deacetylated units) and N-acetyl-glucosamine (acetylated units) [6].

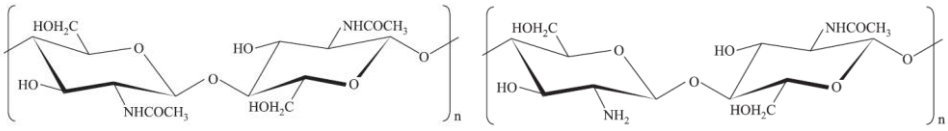

Fig. 1. Chemical structures of: a) chitin and b) chitosan [11].

Chitosan has free amine groups distributed along the chain that allows this polymer to be dissolved in diluted aqueous acidic solutions as acetic acid due to the protonation of these groups [3, 6]. Chitosan presents specific properties as biodegradability, biocompatibility and low-toxicity and has various potential applications in biomedicine, food industry, cosmetics, agriculture, wastewater treatments or biotechnology [2, 7, 12].

There are three methods of chitosan extraction: biological extraction, enzymatic (biotechnological) hydrolysis and chemical process [3, 13]. The most common is the chemical procedure and consists in demineralization, followed by deproteinization, decolorization and deacetylation steps. The process efficiency depends on the temperature used, the alkali concentration and the ratio solution:solid matter of carapace [10, 12].

The purpose of this study is to compare the yield of chitosan extracted from three different sources of Romanian Black Sea Coast and to characterize it in order to evaluate from which species the most qualitative chitosan can be obtained.

2. Materials and methods

2.1 The crab species characteristics

The crustacean samples were collected from two areas of the Romanian Black Sea waters that presented different salinity values. From Midia Port (44°20'2"N/28°40'1"E), located in the Northern area of Romanian seashore, samples of *Carcinus mediterraneus* (Czerniavsky, 1884) were collected in October 2016. From Tuzla (44°00′16″N/28°38′13″E), located in the Southern area of Romanian seashore, the crustacean samples of *Pachygrapsus marmoratus* (Fabricius, 1787) and *Xantho poressa* (Olivi, 1792) were collected in July 2016.

Carcinus mediterraneus (Czerniavsky, 1884) is also known as *Carcinus aestuarii* (Nardo, 1847) [14] or the Green Crab [15] and it is widely distributed in the Mediterranean, Black, Marmara and Azov Seas [14, 16, 17], being particularly common on sandy bottoms, in coastal lagoons, in deltas, under stones, typically in sheltered habitats and in areas where water salinities are lower than those of full seawater [16, 18].

Pachygrapsus marmoratus (Fabricius, 1787) is also known as the Marbled Rock Crab or the Rock Crab and can be found throughout the Mediterranean Sea, including the Black Sea and in North Eastern Atlantic from Brittany (France) southwards to Canary Islands. The Rock Crab is an omnivorous species and a semi-terrestrial crustacean that lives on rocks of upper to middle shore [19, 20].

Xantho poressa (Olivi, 1792) is distributed in the North Eastern Atlantic Ocean, from Portugal to Canary Islands and throughout the entire Mediterranean and Black Seas, at depths from 0 to 15 m. These crabs can mostly be found living under stones [21] and inhabit relatively protected rocky shores, often with pebble underground [22].

More details about the average carapace dimensions, biology, substrat preferences and the distribution of the three species are presented in Table 1.

Table 1

The biological and ecological characteristics of crustacean species

Species	Average carapace dimensions (mm)	Biology and substrat preferences	Distribution
Carcinus mediterraneus syn. *aestuarii* (Czerniavsky, 1884)	65.6 ± 0.84	Intertidal to 10 m (up to 26 m), estuarine and shallow coastal waters, muddy sand, among seagrass, under stones [18]	Mediterranean Sea, Black Sea, Marmara Sea, Sea of Azov, introduced outside of its native Europa in North America's Atlantic and Pacific areas, Australia, South Africa and Japan [14, 16-18]
Pachygrapsus marmoratus (Fabricius, 1787)	33.3 ± 0.43	Intertidal on rocks of upper to middle shore [19, 20]	North Eastern Atlantic and the Mediterranean, including the Black Sea [19, 20]
Xantho poressa (Olivi, 1792)	28.5 ± 0.78	From intertidal to the shallow subtidal zone, near the shore line with rocky substrat (0-15m) [21, 22]	Eastern North Atlantic and South Atlantic Oceans (from Norway and the North Sea south to Morocco) and throughout the entire Mediterranean and Black Seas [21, 23]

The average salinity measured with a VEE GEE handheld refractometer STX-3, type salinity, accuracy ± 1 ‰ with ATC (automatic temperature

compensation) was about 11 ‰ for the Black Sea waters from Midia Port (October 2016) and around 17 ‰ for the waters from Tuzla (July 2016).

2.2 Sample preparation

The collected crabs were imersed in boiling water in order to remove the remaining flesh from the exoscheletons. They were dried overnight at 60 °C, grinded and stored in a dry place until extraction.

2.3 Chitin extraction procedure

All samples collected from Romanian Black Sea were processed using the same procedure. First, the crustacean shells were transformed into powder using a grinder. The chitin extraction procedure was similar to the one presented in [24]. For the first step consisting of demineralization, same concentration of HCl and same ratio solid:HCl as in [24] were used, but the contact time was about 1 h. Because of the high mineral content, low CO_2 emissions were observed when the HCl solution was added. The precipitates were washed with bidistilled water until neutral pH and dried to constant weight. The deproteinization step was carried out using diluted alkaline solution (3 %) NaOH at a ratio of 1:20 (w/v), for 1 h 30 min and for 2 h at 65 °C, under continuous stirring (200 rpm). The difference in time processing for deproteinization was carried out in order to evaluate the time dependence of protein loss. The chitin obtained was washed with bidistilled water until neutral pH and dried until constant weight. In order to remove the impurities and the pigments, the samples were rinsed with acetone and ethanol, and then dried to constant weight.

2.4 Chitin deacetylation process

Chitosan was produced by deacetylation of chitin in a strong alkali solution. Briefly, the obtained chitin was immersed in a 45 % NaOH solution at a ratio of 1:20 (w/v) for 1 h at 95 °C, under continuous stirring (200 rpm). Then, the obtained chitosan was rinsed with bidistilled water until neutral pH was reached and the material was dried to constant weight.

2.5 Chemical composition evaluation

The inorganic salts content was evaluated according to equation (1).

$$\mathrm{IC}\,(\%) = \frac{m_1 - m_2}{m_1} \times 100 \tag{1}$$

where: m_1 = mass of crustacean powder before demineralization (g);
m_2 = mass of crustacean powder after demineralization (g).

The protein content was determined using equation (2).

$$\mathrm{P}\,(\%) = \frac{m_2 - m_3}{m_2} \times 100 \tag{2}$$

where: m_2 = mass of crustacean powder after demineralization (g);
m_3 = mass of crustacean powder after deproteinization (g).

The chitin percentage represents the chitin mass obtained with respect to the crustacean powder mass used for extraction.

2.6 Hygroscopicity evaluation

The moisture of samples kept in atmospheric conditions was determined according to AOAC (2000) [25]. The samples were dried in the oven for 3 hours at 105°C ± 2°C, then they were kept in the dessicator for 30 minutes to cool down. The procedure was repeated until the samples reached constant weight. The moisture was evaluated using the equation (3).

$$\mathrm{M}\,(\%) = \frac{m_i - m_f}{m_i} \tag{3}$$

where: m_i = weight of sample before drying (g);
m_f = weight of sample after drying (g).

2.7 Ash content

The ash content describes the total amount of inorganic material from the samples and it was determined according to F 2103-01 [26]. The analyzed samples were calcinated in a furnace at 800 °C and after cooling down for about 1h, the samples were weighed. Using the equation (4) it was determined the ash content [27].

$$\mathrm{A}\,(\%) = \frac{m_f}{m_i} \times 100 \tag{4}$$

where: m_i = initial weight of sample (g);
m_f = weight of ash (g).

3. Results and discussion

3.1 Samples composition

The composition of the crustacean powders varied with species. The obtained results are presented in Table 2 and they are compared with those obtained for two different species of crustaceans, *Cancer pagurus* (Linnaeus,

1758) and *Chionoecetes opilio* (Fabricius, 1788). These species were selected for comparison due to their similarities concerning the life environment, *C. pagurus* being a crustacean that lives mostly in United Kingdom [28], while *C. opilio* lives in East Sea/Sea of Japan, mostly on the Yeongdeok-gun (South Korea) littoral zone [29].

Table 2

The chemical composition of the samples used for chitin extraction comparative with other crustacean species

Source	Inorganic salts, %	Proteins, %	Chitin, %	Reference
Carcinus mediterraneus (Czerniavski, 1884)	79.80	12.86	7.34	Present study
Pachygrapsus marmoratus (Fabricius, 1787)	83.06	9.49	7.45	Present study
Xantho poressa (Olivi, 1792)	84.35	7.74	7.91	Present study
Cancer pagurus (Linnaeus, 1758)	70.00 - 75.00	13.20 - 17.50	9.70 - 12.00	[28]
Chionoecetes opilio (Fabricius, 1788)	40.60 - 44.16	29.19	26.65	[29]

The Black Sea crustaceans used for extraction reveal a higher inorganic salts content comparing to the other species, but the protein content is lower. These results suggest that the mineral content, as well as the protein content are depending on the marine environment [30].

The highest amount of inorganic salts of the analyzed Romanian Black Sea crustaceans was found in *Xantho poressa*, and the lowest in *Carcinus mediterraneus*. At the same time it can be seen the influence of the water salinity as species of *X. poressa* and *P. marmoratus* are native from the Southern area of Romanian seashore, where the salinity is around 17 ‰, while *C. mediterraneus* lives in the Northern area, where the water salinity is around 11 ‰.

Concerning the chitin composition, it can be seen that the results obtained are similar for the species analysed, but lower than the chitin content in other species. This can be due to the environment conditions but also to the extraction procedures.

The influence of time processing was evaluated depending on the loss of proteins during deproteinization for *Carcinus mediterraneus*. The results are presented in figure 2 where it can be observed that the time has a certain influence on the deproteinization process.

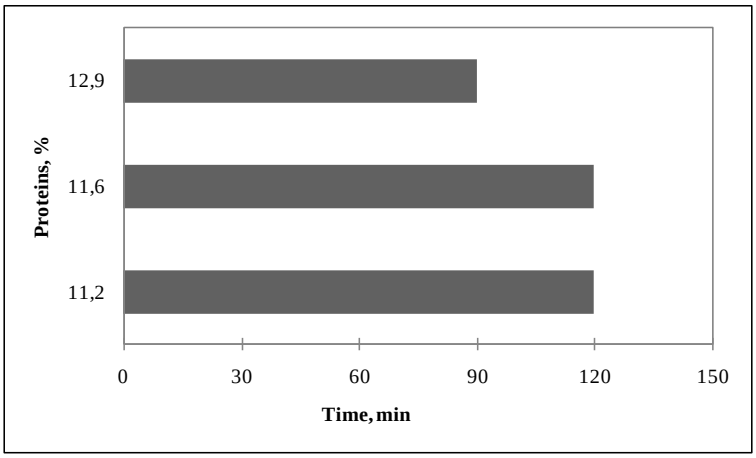

Fig. 2. The influence of time processing on the loss of proteins.

A higher deproteinization time using the same working conditions (identical parameters of alkali concentration, solid:solvent ratio and temperature) led to a higher loss of proteins. Thus, a higher quality chitin and a more efficient extraction procedure could be obtained by varying the deproteinization time.

3.2 Moisture and ash content

The powder from crab species and the obtained chitosan powder were analysed and the results for moisture and ash content are presented in Table 3.

As expected, the ash content is higher in crab powder than in chitosan. The results for ash content (Table 3) showed a higher content of salts for *X. poressa* species compared to *P. marmoratus* and *C. mediterraneus*. Also, the ash content for the chitosan using the same extraction procedures for each species was greater for *X. poressa* and lower for *P.marmoratus*, respectively *C. mediterraneus*.

Table 3

Characteristics of crab powders and chitosan obtained

Source	Crab powders		Chitosan powder	
	Ash (%)	Moisture (%)	Ash (%)	Moisture (%)
Carcinus mediterraneus (Czerniavski, 1884)	37.08 ± 0.22	3.54 ± 0.39	0.44 ± 0.31	5.32 ± 0.34
Pachygrapsus marmoratus (Fabricius, 1787)	44.38 ± 0.61	3.80 ± 0.04	0.53 ± 0.07	5.58 ± 0.64
Xantho poressa (Olivi, 1792)	47.60 ± 0.36	3.44 ± 0.23	1.44 ± 0.37	9.49 ± 0.26

*the results are presented as an average of 3 replicates ± standard deviation.

The values obtained for calcinated crab powders are in agreement with the inorganic content (Table 2) showing that the highest ash content was obtained for the crabs collected from the South area of Romanian Black Sea Coast. The very small values obtained for chitosan ash showed a high efficiency of the demineralization process that led to the production of a high quality chitosan with possible applications in biomedical field [12, 26]. Concerning the hygroscopicity, the results obtained showed that the chitosan presents a higher capacity of water adsorption comparing to the crab powders.

3.3 Spectral characterization

The infrared analysis were performed using a spectrometer model from Interspectrum (Interspec 200-X FTIR Spectrometer) over the frequency range of 4000 ÷ 500 cm^{-1}. Each sample of chitosan was dried before mixing it with previously dried KBr, then pressed to obtain a KBr disc.

The chitosan samples were analysed and compared using a commercial chitosan sample from Sigma-Aldrich. The functional groups and vibration modes are presented in Table 4, where Com-ch represents the commercial chitosan, CM-ch is the obtained chitosan from *Carcinus mediterraneus*, PM-ch is the chitosan obtained from *Pachygrapsus marmoratus* and XP-ch is chitosan produced from *Xantho poressa* species.

In the present study, the main peaks specific for chitosan are represented by the broadband between 3600 ÷ 3300 cm^{-1} which is attributed to O-H and N-H stretching vibrations and by the peaks at 1702.35 cm^{-1} corresponding to C=O stretch in $NHCOCH_3$ group, at 1605.50 cm^{-1} corresponding to NH_2 bending in $NHCOCH_3$ group, at 1420.18 cm^{-1} to OH bending vibrations in CH_2OH group and at 1057.92 cm^{-1} to C-O stretching vibrations.

Table 4

FTIR absorption bands for commercial chitosan and the analysed chitosan samples

Assignements	Wavenumber (cm^{-1}) frequency			
	Com-ch	CM-ch	PM-ch	XP-ch
angular deformation of O-H present in the structure of the chitosan [31] O-H and N-H stretching [32, 33]	3549.05	3547.91	3547.91	3546.26
CH_2 stretch in CH_2OH group [34]	3004.26	3002.40	3005.19	3009.33
C-H stretch in pyranose ring [31, 34]	2958.63	2959.56	2961.43	2961.43
$C = \overset{+}{N}\overset{|}{-}H$ [35]	2209.89	2216.41	2216.41	2214.55
C=O stretch [34]	1702.35	1702.35	1702.35	1707.94
NH_2 bending in amino group [31, 33]	1605.50	1605.50	1605.50	1640.89
OH, CH vibration in the ring [31]	1420.18	1419.25	1419.25	1418.32

OH bending in CH_2OH group [32]				
-NH primary, secondary and tertiary bonds [31]	1360.58	1359.65	1359.65	1359.65
Complex vibrations of NHCO group[34]	1296.32	1298.18	1296.32	1294.46
ν_s (C-O-C) glycosidic linkage [34]	1192.02	1189.23	1187.36	1188.30
ν_{as} (C-O-C) glycosidic linkage [34]	1109.14	1111.00	1112.86	1112.86
C-O stretching [31, 33]	1057.92	1058.85	1056.99	1060.71
Pyranose ring skeletal vibrations [34]	919.16	924.75	921.03	921.03

The molecule vibrations presented in this paper may present shifts in comparison with other studies [31-34]. Peak shifting may be a consequence of variability of sources and of the procedures used for chitin extraction and chitosan preparation [36].

4. Conclusions

By using the same method of chitin extraction and chitosan production for three different types of crabs collected from the Romanian Black Sea Coast it was demonstrated that every species has its own characteristics and the method of extraction needs to be adapted and improved for each species. Although the extraction method was identical for all threee crustaceans, it has led to the production of materials with different properties according to species, in terms of water adsorption capacity, ash content, inorganic salts, proteins and chitin.

The results presented in this paper showed that a good quality chitosan was obtained from *Carcinus mediterraneus* and *Pachygrapsus marmoratus*, while for species of *Xantho poressa* the extraction procedure should be improved in order to decrease the ash content for possible future uses in biomedical field.

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