# IMPROVED METHOD OF CHITOSAN EXTRACTION FROM DIFFERENT CRUSTACEAN SPECIES OF ROMANIAN *BLACK SEA* COAST

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*Extracts of chitin and chitosan from Romanian marine resources have been analysed and compared using spectral methods such as UV-VIS and FTIR analyses, in order to find out a proper marine resource with a higher content of chitosan. These studies are useful for the further investigations related to chitin and chitosan processing in various industrial applications.*

**Keywords**: chitin, chitosan, crustaceans, UV-VIS and FTIR spectroscopy

## 1. Introduction

Marine organisms have been long time recognized as likely to contain many potential biomolecules, due to the environmental conditions specific to their habitat.

Similar to the terrestrial species, marine organisms synthesise a considerable variety of biopolymers, which can be grouped in three main classes: polysaccharides, proteins and nucleic acids. Polysaccharides are biopolymers constituted by carbohydrate monomers (normally hexoses) linked by glycosidic bonds. The most representative polysaccharide in marine environment is chitin.

Chitin (Fig..1a) is a highly crystalline homo polymer, composed by a linear chain of (1→4) linked 2-acetamide-2-deoxy-β-D-glucopyranose units [1, 2]; it is the primary structural component of the exoskeletons of crustaceans and of many other species such as molluscs, insects and fungi. The exoskeleton of

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crustacean samples consists in 30-40% proteins, 30-50% calcium carbonate and 20-30 % chitin, these rates depending on crustacean species. At the same time, it is worth mentioning what extraction methods are used [3, 4] and the period when the samples are collected [5]. Chitin is inert in aqueous environment; thus, its usage is limited.

Chitosan (Fig..1b) [poly-β(1→4)-2-amino-2-deoxy-D-glucose] is produced by the deacetylation of chitin, and it is basic natural polysaccharide which has many attractive properties such as biodegradability, bio compatibility or mucoadhesion, haemostatic action, bacteriostatic and fungistatic activities. These features made it useful in the biomedical, agricultural, environmental, and industrial fields.

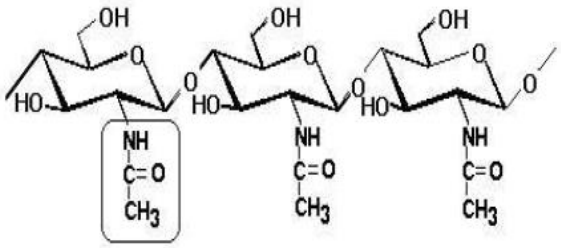


Fig. 1(a) Chitin structure

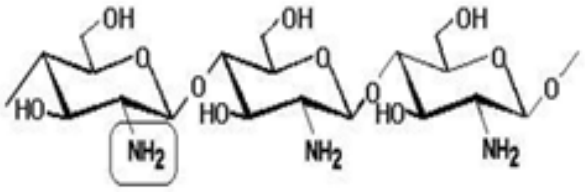


Fig. 1(b) Chitosan structure

Several techniques to extract chitin from different sources have been previously reported [6, 7]. The most common method is referred to as the „chemical procedure" [8, 9]. The chemical method for isolation of chitin from crustacean shell biomass involves two major steps: removal of the inorganic matter (mineral salts) in dilute acidic medium (demineralization) by using HCl, followed by extraction of protein matter in alkaline medium (deproteinization), by treating shell waste with aqueous solutions of NaOH or KOH. The efficiency of alkali deproteinization depends on the process temperature, alkali concentration, and ratio NaOH: shells [10]. The important procedure for obtaining chitosan is based on the alkaline deacetylation of chitin with strong alkaline solution.

The objective of this paper is to present an improved method for chitin and chitosan extraction from various indigenous species of crustaceans from the

Romanian *Black Sea* waters. This proposed method is highly productive in chitosan and consists in using filtration or centrifugal operations as intermediate operations in various steps treatments. Moreover, it is worth mentioning that these indigenous crustacean species have been never studied before from this point of view, fact which opens promising perspectives for the capitalization of these valuable marine bioresurces.

## 2. Materials and methods

The samples of crustaceans were collected in June 2015 from two areas of the Romanian *Black Sea* waters with different salinity. From Cap Midia Gulf (44,3ºN; 28,6ºE), the North area of Romanian seashore, were collected and identified for analysis the following species: *Palaemon elegans* (Rathke, 1843), *Pachygrapsus marmoratus* (Fabricius, 1787) and *Carcinus aestuarii* (Nardo, 1847). Another two crustacean species were collected and identified from Tuzla (44ºN; 028º40'E), located in the South area of Romanian seashore: *Xantho poressa* (Olivi, 1792) and *Eriphia verrucosa* (Forskal, 1775).

The salinity of the waters samples from the two areas mentioned above, were measured with VEE GEE handheld refractometer STX-3, type salinity, accuracy ± 1 ppt with ATC (automatic temperature compensation). In that period, the average value of salinity for the North area was around 12 ppt and for the South area, 16 ppt.

After collection, all the adult specimens of the local crustacean samples were identified [11], weighted and body breadth measured. The specimens with carapace sizes ranging from 2.5 cm to 11.5 cm (Table 1) were selected and for shrimp species (*Palaemon elegans*), the specimens with dimensions of body length ranging from 3.8 cm to 6 cm (Table 1) were selected.

*Table 1*

**Carapace length and areas of the crustacean samples collected**

| Crt. No | Crustacean species | Body dimensions (cm)(min-max) | Weight body(g) (min-max) |
|---|---|---|---|
| 1. | *Palaemon elegans* (Rathke, 1843) (Palaemonidae) | 3.8-6 | 0.5-1.15 |
| 2. | *Carcinus aestuarii* (Nardo, 1847) (Portunidae) | 5.50 – 11.5 | 11-100 |
| 3. | *Pachygrapsus marmoratus* (Fabricius, 1787) (Grapsidae) | 7.5 –10 | 27-45 |
| 4. | *Xantho poressa* (Olivi, 1792) (Xanthidae) | 5.5 - 7.5 | 15-35 |
| 5. | *Eriphia verrucosa* (Forskal, 1775) (Eriphiidae) | 6.0 - 9.5 | 9-85 |

The selected samples were prepared for analysis by washing with a lot of distilled water, then they were soaked in hot water (t=50ºC) for easily detaching of the carapace, claws and tissue. The chemical procedure for extraction process of chitosan from raw crustacean exoskeleton samples consists in three basic steps: demineralization (inorganic salts separation), deproteinization (protein separation) and deacetylation of obtained chitin, according to the standard procedure for chitosan production [10, 12, 13]. In spectral characterization, the reference materials as chitin and chitosan standards were used, from shrimp shells with CAS no. 1398-61-4 and CAS no. 9012-76-4, respectively, purchased from LGC Standards, Germany.

Spectral measurements have been performed using UV – VIS - NIR spectrophotometer Jasco, V 670 model and FT-IR spectrophotometer equipped with ATR device, Perkin-Elmer spectrum 100. UV – VIS – NIR spectra were registered in the range of 200 – 2000 nm, with a step of 0.5 nm.

In all solid samples, IR spectra were recorded between 4000 $cm^{-1}$ and 600 $cm^{-1}$ by accumulation of 32 scans, with a resolution of 4 $cm^{-1}$.

### 2. 1. *Extraction of chitin*

For chitin extraction five sources were used, one kind of shrimps (*Palaemon elegans*) and other four species of crabs from Romanian Black Sea waters.

The carapaces of the collected samples were washed, dried and grounded in very small pieces, to be subjected to demineralization and deproteinization.

The first step: demineralization was carried out at room temperature, using dilute acid solution. The powder of crustacean carapace was soaked in 4% HCl solution (1:15 w/v) for half an hour, under continue stirring (400 rpm). It should be noticed that the emission of $CO_2$ gas indicated the presence of inorganic salts in the samples subjected to extraction. For obtaining high yields in final products, some of the suspensions were filtered by using Buchner funnel in vacuum filtration and other suspensions were centrifugated. Centrifugal operation was realized with Sigma 2K 15 centrifuge, in room conditions.

The precipitates obtained by filtration or by centrifugal operations were washed with distilled water until a neutral pH was obtained, and then all of them were dried up to constant weight.

The second step: deproteinization was performed by soaking the demineralized samples in dilute alkaline solution of 4% NaOH (1:15 w/v), at 65ºC for one hour, under continuous stirring (400 rpm). Some of the suspensions were filtered by vacuum filtration, while the other were centrifugated.

The chitin was obtained in both cases, the precipitates were washed until neutral pH was obtained and then, they were dried up to constant weight. For removing the impurity traces (proteins or other colouring materials), all the

samples of chitin were washed with ethanol solutions and dried up to constant weight. Using centrifugal operation in both steps of chemical extraction, the quantity of obtained chitin is greater than that of chitin obtained by filtration operation. Chitin content was determined by weight difference of the raw crustacean carapace content, subjected to extraction and that of chitin obtained after the demineralization and deproteinization treatments of chemical procedure.

### 2. 2. *Deacetylation of chitin*

The deacetylation was carried out by soaking of dried and purified chitin in concentrate alkaline medium of 45% NaOH solution (1:20 w/v), for 30 minutes, under mild continous stirring (400 rpm). Some of the suspensions of chitosan were subjected to vacuum filtration and other were centrifugated. The wet chitosans obtained were washed with distilled water until neutral pH was reached, and then dried up in oven to constant weight in order to prepare them for spectral characterization.

## 3. Results & discussion

### A. Ecological characteristics of the analysed species

All of the marine invertebrates collected and subjected to chemical procedure are species with ecological characteristics which can be found in Romanian seashore waters of Black Sea. Chitin and chitosan were isolated from five sources of marine invertebrates, from North and South areas of the Romanian Black Sea waters.

*Palaemon elegans* (Ratke 1837) is the ordinary shrimp, being one of euryhalinic and eurybiontic species found on the sandy bottoms covered by algae, [11].

*Pachygrapsus marmoratus* (Fabricius, 1787) is the most common crab on the rocky coasts of the Black Sea, it is omnivore, while *Carcinus aestuarii* **(**Nardo, 1847) can be found on sand and gravel, it is a carnivore species, feeding on many organisms, particularly bivalve molluscs and small crustaceans [14, 15].

*Xantho poressa* (Olivi, 1792) is widely distributed near shore line at a depth less than 1 m, mainly under stones on pebble and crushed rock, rarer on the other bottoms to 15 m. It is verry well adapted to marine environment, it tolerates the cold weather from winters and feeds itself with fish carrions.

*Eriphia verrucosa* (Forskal, 1775) is the biggest crustacean from the Black Sea waters, which lives among stones and seaweeds in shallow water along rocky coastlines up to a depth of 15 m [17]. It is a carnivore species, feeding on bivalves, gastropods [18], or on molluscs [15, 16, 17].

### B. Chemical structure of the marine invertebrates exoskeletons studied

The results obtained by experimental works indicated that the exoskeletons chemical structure is distinct for each species. The differences appear according to the treatment used in the chemical extraction.

As it is shown in Figs. 1 and 2, the shrimps species (*P. elegans*) had a higher content of proteins (68% and 57%, respectively) in their exoskeletons relative to the other crustacean species studied.

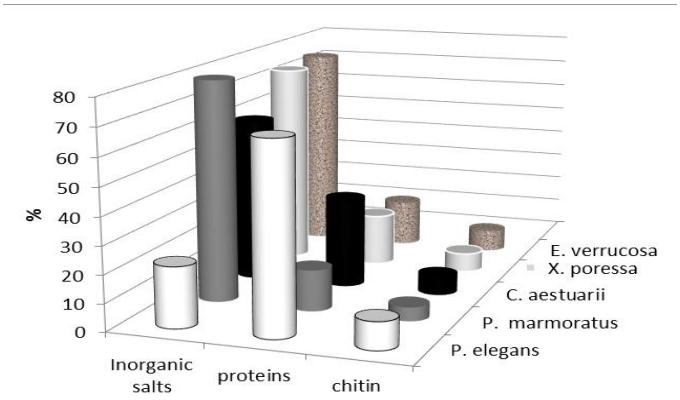


Fig. 1 Chemical composition of the studied samples- obtained using filtration

The highest content of inorganic salts has been found in the exoskeletons of *P. marmoratus* (80% and 79%), a crab species from the North area of the Romanian Black Sea waters, followed by the crab species from the South area: *X. poressa* (74% and 77%) and *E. verrucosa* (75% and 70%). The exoskeleton of the shrimp species (*P. elegans*) had the highest content of chitin (13%), followed by the crab species as: *C. aestuarii* (11%) and *E. verrucosa* (10%).

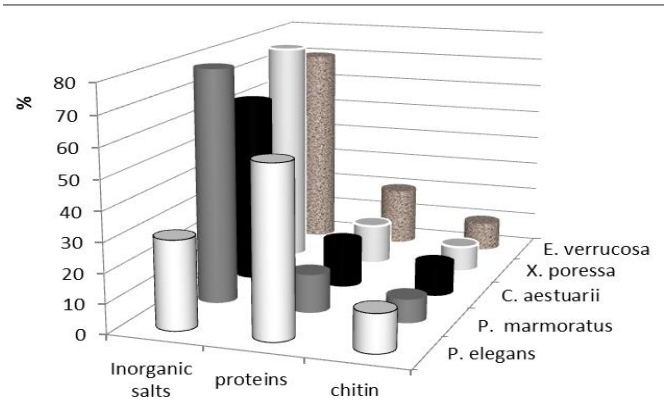


Fig. 2 Chemical composition of the studied samples - obtained using centrifuging

The use of centrifugal operation in the steps of the chemical procedure for slurry separation is more indicated than filtration to obtain a higher efficiency in final products: chitin and chitosan (Figs. 3 ÷ 5).

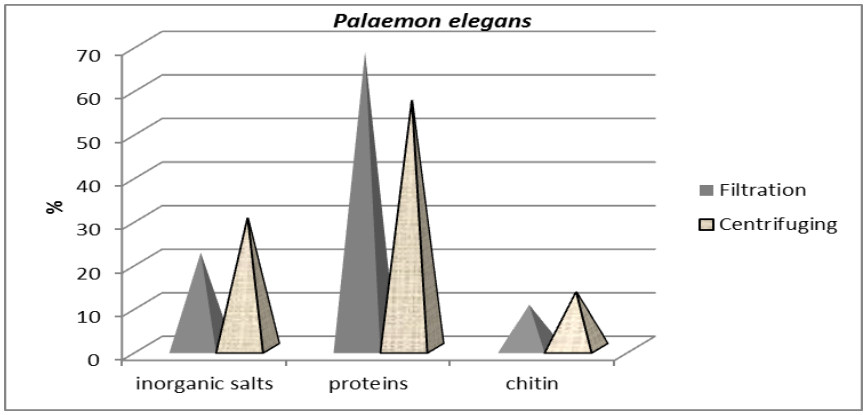


Fig. 3 Chemical procedure efficiency for *P.elegans* samples

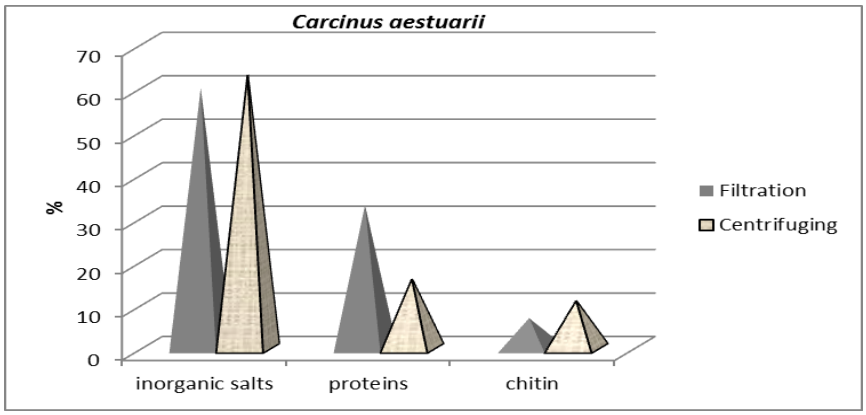


Fig. 4 Chemical procedure efficiency for *C.estuarii* samples

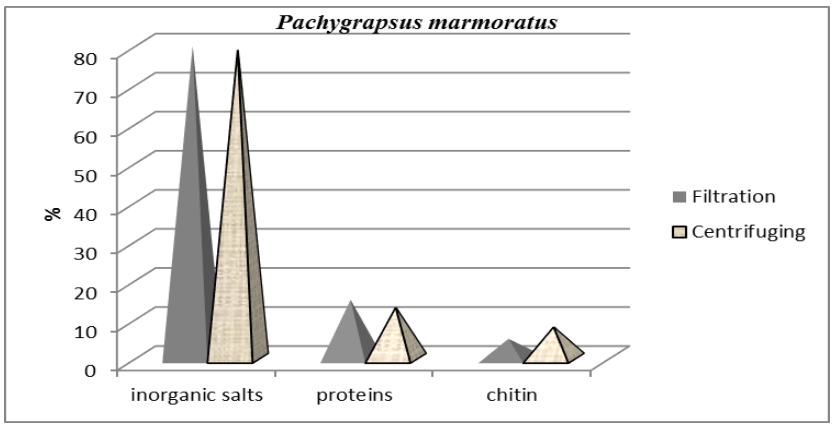


Fig. 5 Chemical procedure efficiency for *P.marmoratus* samples

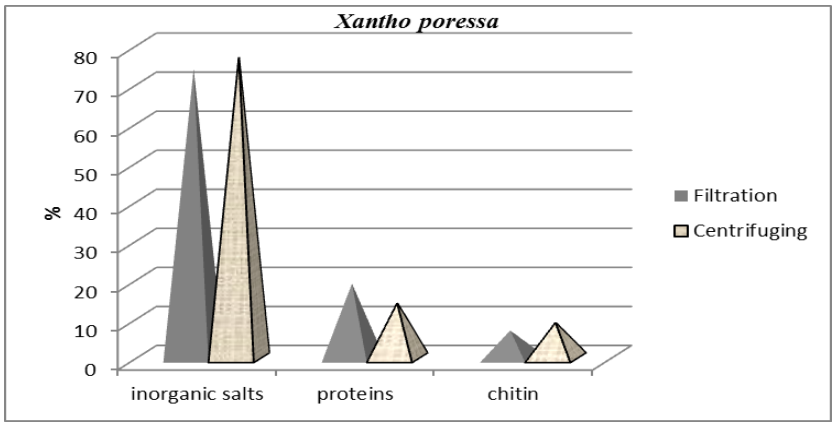


Fig. 6 Chemical procedure efficiency for *X.poressa* samples

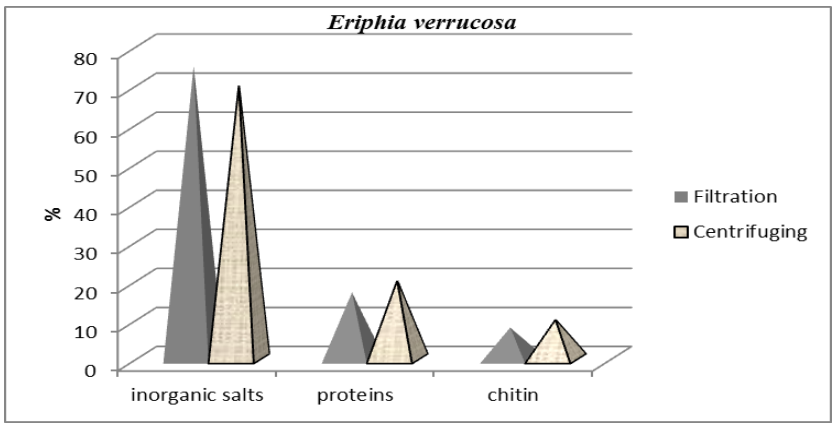


Fig. 7 Chemical procedure efficiency for *E. verrucosa* samples

## C. Spectral characterization of chitin and chitosan extracted from local sources

It is known that chitin and chitosan, like most of polymers and biopolymers, absorb in UV [19, 20]. The obtained results from UV-VIS-NIR spectroscopy of the chitin and chitosan, extracted from local crustacean species studied are presented in Fig. 8. The electronic spectra recorded for the chitin obtained from the one source of crustaceans (*P. elegans*), using centrifuging were compared with the UV – VIS – NIR spectrum of standard chitin (Fig. 8), while the UV-VIS-NIR spectra recorded for the chitosan obtained from the other crustacean sources (*X. poressa*), using centrifuging were compared with the UV-VIS-NIR spectrum of standard chitosan (Fig. 8).

The presence of chitosan in the sources studied is confirmed by the one of two absorption peaks at 210 and 250 nm.

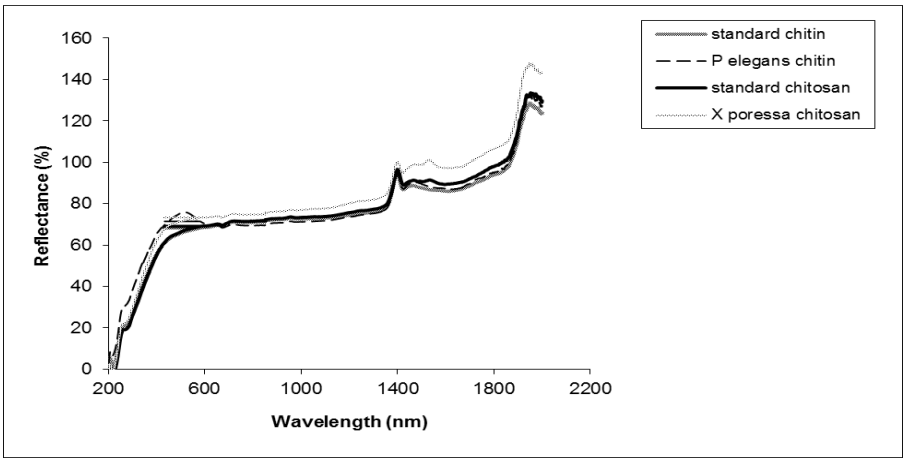


Fig. 8 UV-VIS-NIR spectra of chitin and chitosan samples

Different absorption bands were registered in 4000 ÷ 600 $cm^{-1}$ area in the IR spectra of chitin and chitosan samples obtained from two different crustaceans sources, using centrifuging and compared to those of standard chitin and chitosan (Fig. 9).

The IR spectra of chitin and chitosan exhibit characteristic bands in 3450 ÷ 3200 $cm^{-1}$ domain, attributed to stretching vibration of – OH groups overlapped to the stretching vibration of N – H. The more intensive absorption bands are obtained for standard chitin and *P. elegans* chitin and the bands became wider in the case of standard chitosan and *X. poressa* chitosan, this behaviour being attributed to a deacetylation process from chitin to chitosan [21].

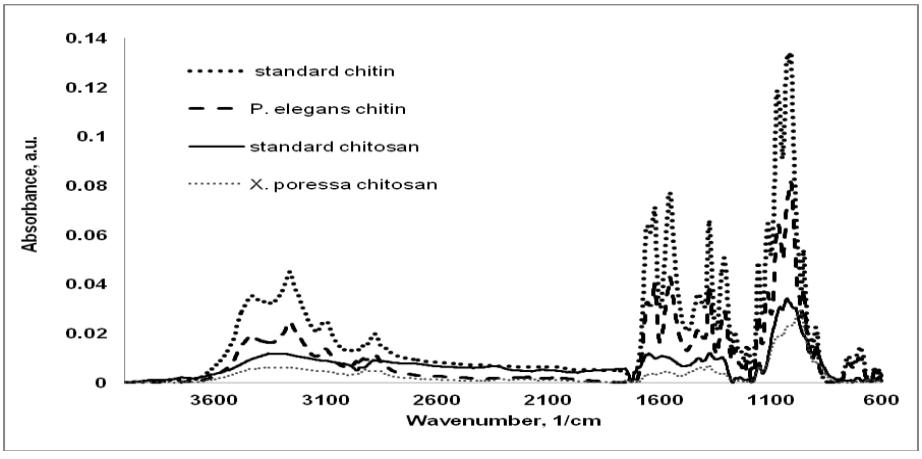


Fig. 9 FT-IR/ATR spectra of chitin and chitosan samples

Other absorption bands, used in the literature as characteristic bands to analyze chitin and chitosan, are located between 2960 $cm^{-1}$ and 2850 $cm^{-1}$, being assigned to the valence vibrations of the C – H bondings as $\nu_{as\ C-H}$ in – $CH_2$ – and $\nu_{sC-H}$ in – $CH_3$. The strong peaks corresponding to a larger amount of C-H bonds are registered for standard chitin and *P. elegans* chitin, and they decreased for standard chitosan and *X. poressa* chitosan. Comparing all these spectra, it could be estimated that the lowest intensity of this absorption band is related to the highest deacetylation level of the sample.

The bands observed at 1655 ÷ 1620 $cm^{-1}$ indicate a stretching vibration mode of the amidic group. The presence of carbonyl group of Amide I [22] corresponds to the incomplete deacetylation of chitin to chitosan. The most intensive band of Amide I is obtained for standard chitin and decreased for *P. elegans* chitin. A visible decreasing in the intensity of these bands occurred for chitosan samples, *X. poressa* chitosan showing a more advanced deacetylation step, by removing the acetyl groups from chitin structure. Specific absorption bands for N-acetylglucosamine appear at around 1375 $cm^{-1}$ corresponding to -$CH_3$ symmetric deformation [23, 24]. The more intensive peak at 1376 $cm^{-1}$ is registered in standard chitin and decreases for *P. elegans* chitin. In the case of chitosan samples, a strong decreasing in the intensity of this band is noticed, from standard chitosan and *X. poressa* chitosan.

Absorption in the range from 1160 $cm^{-1}$ to 1000 $cm^{-1}$ are attributed to –C–O–C in glycosidic linkage and to $\nu_{C-O}$ in primary OH group, respectively. These bands are visible in all the spectra, the intensity decreasing from standard chitin to *P. elegans* chitin, standard chitosan and to *X. poressa* chitosan.

The presence of the absorption bands in 895 ÷ 850 $cm^{-1}$ domain in all the spectra also indicates the polysaccharides structure.

## 4. Conclusions

It was demonstrated that it is possible to extract chitin and chitosan from different crustacean sources of the Romanian Black Sea Coast by infrared and UV –VIS – NIR studies.

In order to obtain a proper efficiency in chitosan, it is necessary to improve the chemical procedure of extraction, by using the best and adequate methods. In our case, the centrifuging is indicated for separation of the slurry obtained.

Crustacean decapods sampled from the Romanian Black Sea waters are a rich source of chitin and chitosan, which make them useful in various applicative fields, ranging from waste treatment or ballast water management to biotechnology and medicine.

# R E F E R E N C E S


[1] *M. N. V. R. Kumar*: 'A review of chitin and chitosan applications', React. Funct. Polym., 2000, **46**, (1), pp:1–27.

[2] *Ben Amar Cheba*, „Chitin and Chitosan: Marine Biopolymers with Unique Properties and Versatile Applications", Global Journal of Biotechnology & Biochemistry ***6*** (3), 2011, pp: 149-153,

[3] Y.I. Cho, H.K. No and S.P. Meyers, "Physicochemical Characteristics Chitin and Chitosan Production". J. Agric. Food Chem., 46, 1998, pp. 3839-3843.

[4] H.K. No, K.S. Lee and S.P. Meyers; "Correlation between Physicochemical Characteristics and Binding Capacities of Chitosan Products". J. Food Sci., 65, pp. 1134-1137, 2000.

[5] *Green JH, Mattick JF*. "Fishery waste management". In: J.H. Green and A. Kramer (Eds). Food processing waste management. AVI publishing, West port, UK, pp. 202- 307, 1979.

[6] *Wassila Arbia, Leila Arbia, Lydia Adour, Abdeltif Amrane*, „Chitin Extraction from Crustacean Shells Using Biological Methods – A Review", Food Technol. Biotechnol. 51 (1) 12–25, 2013.

[7] *S. Das, E.G. Anand Ganesh,* „Extraction of chitin from trash crabs (Podophthalmus vigil) by an eccentric method", Curr.Res. J. Biol. Sci. 2, pp 72–75, 2010.

[8] *Suneeta Kumari, P. Rath , A. Sri Hari Kumar, T.N. Tiwari,* Extraction and characterization of chitin and chitosan from fishery waste by chemical method, Environmental Technology & Innovation 3, 2015, pp 77–85

[9] *Abdulwadud A, Muhammed TI, Surajudeen A, Abubakar JM, Alewo OA*. Extraction and characterisation of chitin and chitosan from mussel shell. Civ Environ Res. 2013;3:109–14.

[10] *F.A. Al Sagheer, M.A. Al-Sughayer , S. Muslim, M.Z. Elsabee*: „Extraction and characterization of chitin and chitosan from marine sources in Arabian Gulf'", Carbohydrate Polymers 77, 2009, pp: 410–419

[11] *M. Bacescu*, – Fauna RSR Crustacea, Decapoda, **vol. IV**, fasc.9, 1967, Ed. Academiei, pp: 97-113

[12] *H.K. No & S.P. Meyers*, "Preparation and Characterization of Chitin and Chitosan – A review.", J Aquatic Food Prod. Technol., **4,** 1995, pp. 27- 52

[13] *E.S. Abdou, K.S.A. Nagy, and M.Z. Elsabee*, „Extraction and characterization of chitin and chitosan from local sources", *Bioresource Technology*, **Vol. 99** (5), 2008, pp. 1359–1367

[14] ****"European green crab". Washington Department of Fish and Wildlife.* Archived from the original on June 13, 2008. Retrieved February 12, 2010 (accessed March 2016).

[15]. *D. Atodiresei, V. Chitac, M. Pricop, C. Pricop, M. T. Onciu* - Influence of noise on the physiological activity of the blue mussel (*Mytilus Galloprovincialis*) from the Black Sea, Constanta Maritime University's Annals, ISSN 1582-3601 **vol. 18**, 2012, pp: 89 – 94.

[16]. *D. Atodiresei, V. Chitac, M. Pricop, F. Nicolae, A. Toma, I. Scurtu* „NS MIRCEA" compartments classification and noise analysis of the marine environment by acoustic emissions. "Mircea cel Batran" Naval Academy Scientific Bulletin, **vol. XVII** ( 1), pp. 13-17, 2015.

[17] ****Eriphia verrucosa*". European Virtual Aquarium. Retrieved January 27, 2009 (accessed March 2016).

[18] *A. C. Rossi & V. Parisi* "Experimental studies of predation by the crab *Eriphia verrucosa* on both snail and hermit crabs occupants of conspecific gastropod shells".Bollettino di Zoologia, **40,** 1973, pp: 117–135.

[19]. *M. R. Apetroaei, A M. Manea, G. T. Tihan, R. G. Zgârian, V. Schroder, G. Lilios, G. M. Apetroaei, I. Rău*, "Chitosan an eco-friendly biomaterial from marine invertebrates". *The 5th IEEE International Conference on E-Health and Bioengineering - EHB 2015*

[20]. *M. R. Apetroaei, R.G. Zgârian, A.M. Manea, I.Rau, G. T. Tihan, V. Schroder*, „New source of Chitosan from Black Sea marine organisms identification", *Molecular Crystals and Liquid Crystals*, vol. 628, 2016, pp. 102-109

[21] *T. Muslim, M. Habibur R.Hosne, Ara Begum, Md. Azizur Rahman* „Chitosan and Carboxymethyl Chitosan from Fish Scales of Labeo rohita" Dhaka Univ. J. Sci. **61**(1), 2013, pp: 145-148.

[22] *P. Petrache, S. Rodino, M. Butu, G. Pribac, M. Pentea, M. Butnariu*. – "IR spectroscopy of the flour from bones of European hare". Dig J Nanomater Bios. 9(4): 1523, 2014, pp 23.

[23] *DONG Yanming, XU Congyi, WANG Jianwei, WANG Mian, WU Yusong, RUAN Yonghong*, "Determination of degree of substitution for N-acylated chitosan using IR spectra", *SCIENCE IN CHINA*, **Vol. 44**, No. 2, 2001.

[24] *Sânia M. B. de Andrade, Rasiah Ladchumananandasivam, Brismak G. da Rocha, Débora D. Belarmino, Alcione O. Galvão, "*The Use of Exoskeletons of Shrimp (*Litopenaeus vanammei*) and Crab (*Ucides cordatus*) for the Extraction of Chitosan and Production of Nanomembrane", Materials Sciences and Applications, **3**, 495-508, 2012.