Indian Journal of Geo Marine Sciences
Vol. 51 (04), April 2022, pp. 304-309

Identification of $CaCO_3$ polymorphs of shellfish by FTIR spectroscopy and evaluation of metals adsorption by powdered exoskeleton shell

K S Singh* & S G Sawant

Bio-organic Chemistry Laboratory, CSIR-National Institute of Oceanography, Dona Paula, Goa – 403 004, India

*[E-mail: keisham@nio.org; keisham.sarjit@gmail.com]

Received 17 December 2020; revised 05 April 2022

Shellfish are found in the coastal region throughout the tropical, sub-tropic and temperate zone. A variety of shellfish species are known in nature. Being filter-feeding organisms, organic and inorganic substances easily gain entry into them. Very often, heavy metals such as Cd, Pb, Cu, and even Hg can accumulate in their bodies. The presence of these heavy metals in shellfish is an indicator of environmental pollution, and a threat to humanity as these fish are widely consumed in coastal regions. Here, biochemical analysis of exoskeleton shell mainly composed of polysaccharides, proteins and calcium carbonate ($CaCO_3$) - have been studied. Calcium carbonate ($CaCO_3$) which is one of the major constituent of the exoskeleton part of the shellfish exists in three polymorphs *viz*., calcite, aragonite and vaterite. The identification of $CaCO_3$ polymorphs in shellfish is extremely important as it gives information on the structural arrangement of the calcium carbonate lattice in these organisms. Here, we present a convenient method for the identification of $CaCO_3$ polymorphs in shellfish collected from Goa, west coast of India, with Fourier-Transform Infrared Spectroscopy (FTIR) and further confirmed by powder X-ray analysis. Additionally, the potential of the powdered shell biomass for metal removal from an aqueous medium has also been investigated.

[**Keywords**: Bio-mineralization, Calcium carbonate, Infrared spectroscopy, Polymorph, Shellfish]

Introduction

Shellfish are commonly found in the coastal regions, and their various species are known in nature. They do not only form an essential cuisine for coastal people but are also a source of nutrients. Being filter-feeding organisms, organic and inorganic substances can easily enter their body leading to the accumulation of toxic materials, such as organic pollutants and heavy metals, including Cd, Pb, Hg, etc. The bioaccumulation of these compounds is an immense threat to human beings as a vast population in the coastal region feed on shellfish. The exoskeleton of shellfish is chiefly composed of polysaccharides, such as chitin, chitosan, proteins and calcium carbonate ($CaCO_3$). Intriguingly, several marine organisms, including shellfish, produce $CaCO_3$ using a sophisticated bio-mineralization process[1,2], and it was claimed that biologically produced $CaCO_3$ have superior mechanical properties compared to the naturally occurring $CaCO_3$(refs. 3,4). The biomineralization of $CaCO_3$ by marine organisms is vital in the carbon cycle for the fixation of carbon dioxide from the atmosphere. Further, the mechanical properties of $CaCO_3$ lattice depend on several factors, *e.g*. size and arrangement of crystal units, polymorph composition (calcite/aragonite ratio), degree of calcium substitution in $CaCO_3$ by Mg and Sr, and the quality and quantity of organic matrix[5,6]. Although mineralogy in shells is mainly biologically controlled, the chemistry and environmental factors also play an essential role. Indeed, $CaCO_3$ crystal size and structure are partially determined by environmental factors, including pH, salinity and temperature[7,8]. For instance, a higher water temperature leads to enhanced aragonite/calcite proportion, while low salinity reduces its proportion[9,10]. On the other hand, a decrease in pH in the environment can alter the composition and mechanical properties of shellfish[11].

$CaCO_3$ exists in three significant polymorphs, *viz.* aragonite, calcite and vaterite, of which aragonite and calcite are more stable and commonly found in shells. Calcite has trigonal symmetry, whereas aragonite has orthorhombic symmetry, a compact structure composed of triangular carbonate ion groups (CO_3^{2-}), with carbon at the centre of the triangle and three oxygen atoms at each corner. Aragonite can be transformed into more stable calcite under physical and chemical conditions. In nature, aragonite is readily converted into calcite at a temperature above 380 °C(ref. 12). Previously, Checa *et al.* reported that by enrichment of Mg in seawater, calcite nature of carbonate in bivalves could tune to aragonite[13].

Numerous methodologies are utilised for identifying $CaCO_3$ polymorphs, such as FTIR[14], X-ray diffraction[15] and surface analysis techniques, namely XPS and TOF-SIMS[16]. Although the FTIR technique is often used for the identification of the carbonate polymorph of $CaCO_3$[17], its utility in the identification of the carbonate polymorph present in shellfish is scarce. The swift and cost-effective identification of the $CaCO_3$ structure is necessary to understand the nature of carbonate allotropy of shells as it could correlate to the metal accumulation in the shellfish exoskeleton. For instance, aragonite had higher adsorption affinity towards Cd^{2+}, whereas calcite had higher adsorption capacity toward Pb^{2+}(refs. 18,19). Thus, structural information of the shell exoskeleton could give preferential metals that could accumulate into the shell biomass.

An extensive study was carried out on the biochemical analysis of shellfish to understand their nutritional value[20,21]. Moreover, the application of chitosan extracted from shellfish exoskeleton has been studied in various domains, such as catalysis[22], and materials and biomedical applications[23,24]. However, a limited study is done on the application of shell biomass for removing metals and organic contaminants. In 2014, Wu *et al.*[25] reported the removal of Cu by the exoskeleton of waste oyster shells. In 2019, aragonite crystals synthesized from egg shells were employed for the efficient removal of heavy metals, such as cadmium and lead[26]. Heavy metals removal by waste shell biomass is an attractive and viable method as shell wastes are cheap and readily available. Furthermore, finding a sustainable use of these shell wastes could help in waste management through routine harvesting of the biomass.

In the present study, a fast and cheap identification method for $CaCO_3$ polymorphs, present in shellfish collected from Goa, has been described. Moreover, the exoskeleton biomass of shells has been investigated for metal adsorption from an aqueous solution.

Materials and Methods

The **s**hells used in this study were either collected from the Goa coast or obtained from the local market. Shellfish studied in this study include Oyster (*Villortia cyprinoids*), Clam (*Meretrix casta* and *Perna erosa*), molluscs (*Perna viridis*), gastropods (*Bolinus brandaris*) and an unidentified gastropod *sp*. In the case of clams, 2-3 organisms were sampled, whereas for other organisms, only one sample of each organism was analysed. Shells were thoroughly washed with water, and the exoskeleton was separated from the soft body matrix. The exoskeleton was treated with dilute HNO_3 (5 %), washed several times with distilled water and finally, dried in a hot air oven. The dried exoskeleton was grounded to powder by a mechanical rotor. The powder was directly used for FTIR and metal adsorption studies.

FTIR spectroscopy

FTIR spectra were recorded in the 400 – 4000 cm^{-1} spectral region at room temperature using about 1 mg of sample mixed with KBr (1:20) and grounded into powder in a mortar. Spectra were recorded at 4 cm^{-1} resolution with 20 scans on a Shimadzu Infinity-I infrared spectrophotometer equipped with Diffuse Reflected Spectroscopy assembly, whereas pH and the Total Dissolved Solids (TDS) were measured using the OKTON-510 bench top instrument.

Determination of metal adsorption by shell biomass

1 g of the salt was dissolved in 1000 ml of distilled water. Then 50 ml of each of the metal solutions were added to 1 g of the grounded calcite/aragonite sample in a 100 ml conical flask. The flasks were covered and kept aside for 2 days. After 2 days, the solution was filtered. The pH and TDS were recorded for the standard metal solution as well as the solution obtained after filtration through a column packed with shell biomass.

Results and Discussion

FTIR spectroscopy was used to identify $CaCO_3$ polymorphs present in the shellfish collected from the Goa coast. FTIR is a powerful tool for the identification of allotropes of crystalline carbonate *viz*. calcite, aragonite and vaterite[27]. Usually, calcite structure showed IR absorption frequencies for the carbonate ion at around 1087 (ν1), 881 (ν2), 1432 (ν3) and 713 (ν4) cm^{-1}. In the case of aragonite, the absorption at 713 (ν4) split into two peaks at 710 and 714 cm^{-1}, which is characteristic of the aragonite structure, whereas for vaterite absorption peak for ν4 appeared at 741 cm^{-1} (Table 1)[17].

In this work, the dried and powdered exoskeleton of shellfish was used to study the carbonate stretching frequency in the mid-IR region. Figure 1(a, b) showed

Table 1 — Fourier transform stretching frequency (wave numbers in cm^{-1}) of carbonate ion for calcite, aragonite and vaterite[a]

S No.	Calcite				Aragonite				Vaterite				Ref.
	*v*1	*v*2	*v*3	*v*4	*v*1	*v*2	*v*3	*v*4	*v*1	*v*2	*v*3	*v*4	
1		874	1430	710	1080	855	1470	710 698					Nakamoto *et al.*[31]
2	1087	881	1432	712	1087	866	1550	715 703	1090	878	1450	741	Weir *et al.*[32]
3	870	1430	1430	715	1080	855	1475	715 700	1085	870	1490 1420	750	Sato *et al.*[33]
4	1079	876		713	1081	853		712 694					Donoghue *et al.*[34]

[a]Source: Anderson & Brecevic (1991) references therein[17]

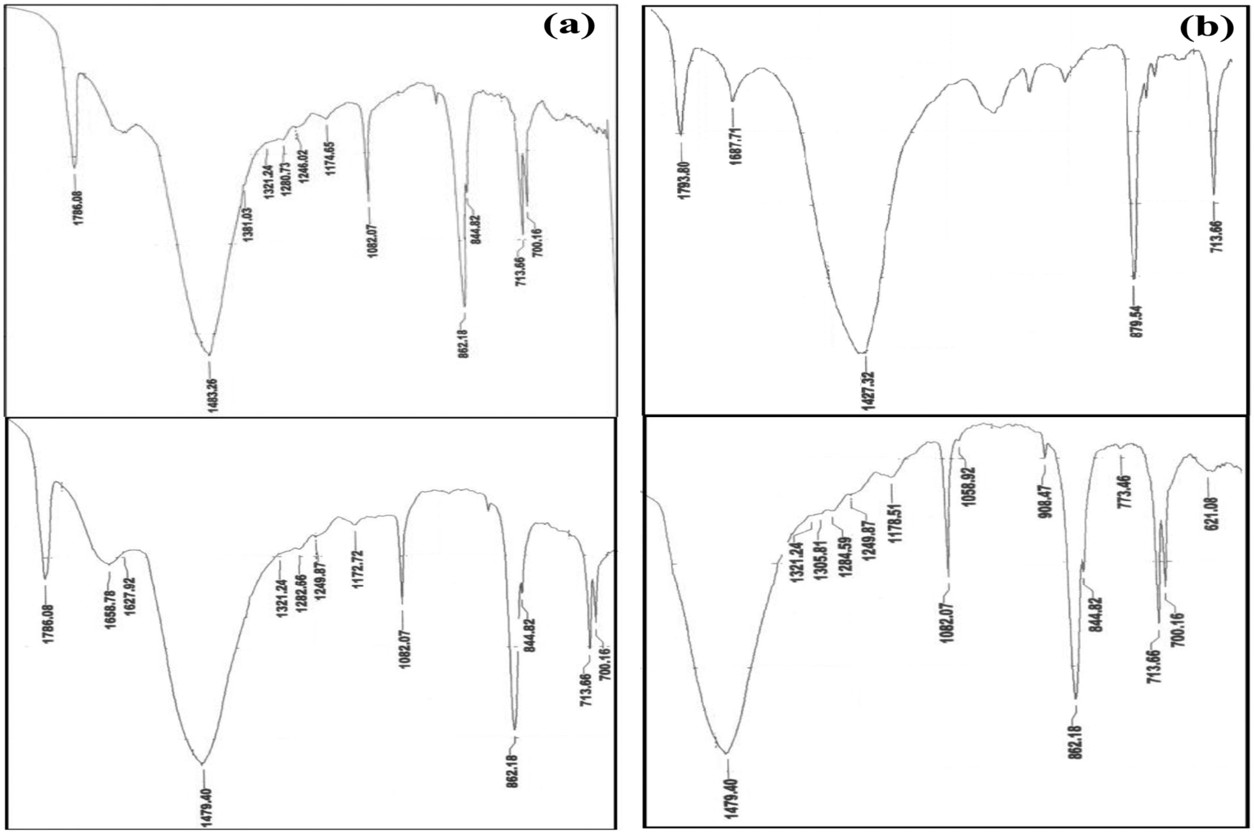

Fig. 1 — a) FT-IR spectra of: Clam (*Meretrix casta*) (up), Mollusc (*Perna viridis*) (down); and b) FT-IR spectra of: oyster (*Villortia cyprinoids*) (up), Gastropod (*Stramonita haemastoma*) (down)

the FTIR spectra of shellfish in the carbonate stretching frequency region. The significant absorption frequencies (cm^{-1}) of shells under investigation are summarized in Table 2. The FTIR spectrum of powdered oyster (*Villortia cyprinoids*) contained two broad bands at 1083 and 877 cm^{-1} assignable to ν(1) and ν(2), respectively. The strong absorption band at around 1427 cm^{-1} and a weak band at 713 cm^{-1} are due to the normal mode of vibrations, v3 and v4, respectively. The spectrum showed the characteristic calcite band for v2 at 877 cm^{-1}, and a single band for v1 at 713 cm^{-1}, confirming the calcite nature of carbonate polymorph for *Villortia cyprinoids*.

In the case of clam, *Meretrix casta* and *Perna erosa*, the FTIR spectrum displayed characteristic aragonite absorption bands at 862 cm^{-1} and 864 cm^{-1}, respectively and a weak peak corresponding to v1 vibration appeared at around 712 cm^{-1}, which split into two bands (711 and 700 cm^{-1}). Similarly, the FTIR spectra of *Perna viridis*, *Bolinus brandaris* and the unidentified gastropod showed a similar pattern of the

Table 2 — The infrared peaks for various shells collected from Goa in KBr (500-4000 cm^{-1} ranges)

No.	Name of edible shell	IR Band (cm^{-1})	*Asymm.* and bending frequency of CO_3^{2-} (cm^{-1})	Allotrope type of carbonate
1	Oyster (*Villortia cyprinoids*)	2513, 1795, 1685, 1512, 1427, 1159, 1083, 1010, 877, 713	1427, 1083, 1010, 877, 713	Calcite
2	Clam (*Meretrix casta*)	2546, 2520, 2497, 1786, 1483, 1082, 862, 844, 713, 700	1483, 1082, 862, 844, 713, 700	Aragonite
3	Clam (*Perna erosa*)	2546(m), 2520(m), 2497(m), 1786(s), 1435(s), 1082(m), 864(s), 844(w), 713(s), 700(m)	1483, 1082, 864, 844, 713, 700.	Aragonite
4	Mollusc (*Perna viridis*)	2546(m), 2520(m), 2497(m), 1786(s), 1479(s), 1082(m), 862(s), 844(w), 713(s), 700(m)	1479(s), 1082(m), 862(s), 844(w), 713(s), 700(m)	Aragonite
5	Gastropod*	2546, 2520, 2497, 1786, 1481, 1082, 862, 844(vw), 711, 700	1481, 1082, 862, 844(vw), 711, 700	Aragonite
6	Gastropod (*Bolinus brandaris*)	2542, 2520, 2498, 1786(m), 1483 (s), 1082(m), 862(m),713(m) and 700 (m)	1483(s), 1082(m), 862(m), 713(m) and 700 (m)	Aragonite

*Species not identified; Abbreviations: s - strong; m - medium; w - weak; vw - very weak

FTIR bands confirming the carbonate present in them is of aragonite polymorph (Table 2).

The polymorph nature inferred from FTIR spectra was corroborated by powder XRD analysis. Powder XRD of $CaCO_3$ crystals exhibited prominent peaks of 2θ at 29.4° for calcite, whereas aragonite exhibited two strong peaks of 2θ at 26.2° and 27.2°, respectively[28]. In the powdered X-ray pattern of the oyster sample, *Villortia cyprinoids* exhibited a strong peak for 2θ at 28.6° (Fig. 2), on the other hand, clam sample, *Meretrix casta* showed two peaks for 2θ at 26.7° and 28.2° (Fig. 3) confirming that $CaCO_3$ polymorph present in *Villortia cyprinoids* is calcite and that of *Meretrix casta* is aragonite. The powder XRD graphics of *Villortia cyprinoids* and *Meretrix casta* are shown in Figures 2 & 3, respectively. The XRD pattern of *Villortia cyprinoids* and *Meretrix casta* are in agreement with the calcite and aragonite structures[29,30].

The metal absorption property of the exoskeleton powder was investigated by measuring the pH and TDS of the standard solution (control) and solution after treatment with shell powder. In a typical experiment, the pH of salt solutions, namely, $FeCl_3$ (anhy), $Fe(NO_3)_3.9H_2O$, $Cu(NO_3)_2.3H_2O$, $CdCl_2.H_2O$ and $Pb(NO_3)_2$ were measured before and after the treatment with the shell powder, *viz.* calcite and aragonite carbonates. As evident from Table 3, the pH of $FeCl_3$ (anhy.) standard solution (pH ≈ 2.65) increased to 7.97 and 7.94, post-treatment with the shellfish powder oyster (calcite) and clam (aragonite), respectively, indicating a significant absorption of iron by the shell powder. The increase in the pH was most significant for $FeCl_3$. (anhy.) and the least for $Pb(NO_3)_2$ (Table 3). The metal absorption property of the shell powder was also studied by measuring the TDS of the standard solution and the metal solution after treatment with the shell powder (calcite and aragonite). TDS of the metal solution decreased

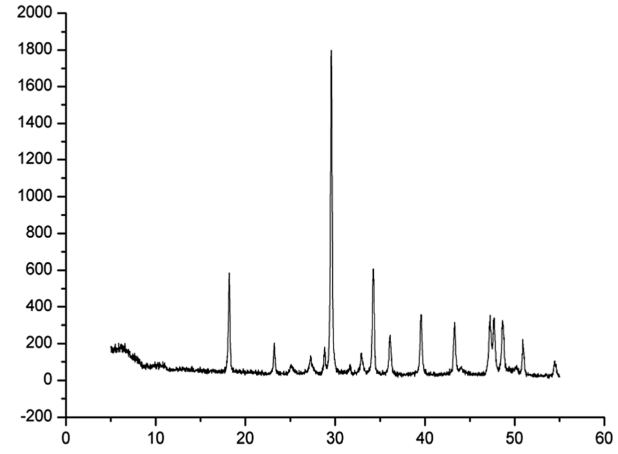

Fig. 2 — Powdered XRD of the exoskeleton of oyster (*Villortia cyprinoids*)

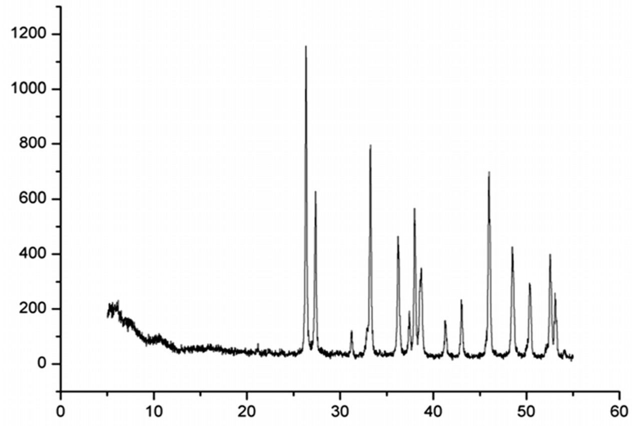

Fig. 3 — Powdered XRD of the exoskeleton of clams (*Meretrix casta*)

Table 3 — Result of pH of metal solutions before and after treatment with shell powder

Metal solution	pH of the standard solution			pH of the solution after treatment					
				Calcite			Aragonite		
	Reading 1	Reading 2	Mean	Reading 1	Reading 2	Mean	Reading 1	Reading 2	Mean
$FeCl_3$	2.64	2.66	2.65	7.97	7.96	7.97	7.95	7.93	7.94
$Fe(NO_3)_3.9H_2O$	3.01	3.03	3.02	8.03	8.05	8.04	8	8.01	8.01
$CuNO_3.3H_2O$	5.38	5.37	5.38	5.97	5.95	5.96	5.98	5.99	5.99
$CdCl_2.H_2O$	5.63	5.61	5.62	7.25	7.27	7.26	7.84	7.83	7.84
$Pb(NO_3)_2$	4.98	4.98	4.98	7.66	7.62	7.64	7.67	7.69	7.68

Table 4 — Result of TDS of metal solutions before and after treatment with shell powder[a]

Metal solution	TDS of the standard solution			TDS of solution after treatment					
				Calcite			Aragonite		
	Reading 1	Reading 2	Mean	Reading 1	Reading 2	Mean	Reading 1	Reading 2	Mean
$FeCl_3$	5.938	5.939	5.939	2.882	2.88	2.881	2.843	2.846	2.844
$Fe(NO_3)_3.9H_2O$	3.307	3.306	3.307	1.537	1.538	1.538	1.47	1.473	1.472
$CuNO_3.3H_2O$	1.227	1.228	1.228	1.218	1.219	1.219	1.216	1.216	1.216
$CdCl_2.H_2O$	1.227	1.228	1.228	1.103	1.105	1.104	1.108	1.108	1.108
$Pb(NO_3)_2$	1.807	1.807	1.807	1.058	1.059	1.059	1.012	1.011	1.011

[a]TDS values of solutions are in ppm

significantly from the corresponding standard solution after treatment with the shell powder (Table 4). The results showed that the exoskeleton of shellfish, which is usually discarded, is good metal absorber. Hence, the exoskeleton of shell has the potential as an alternative method to remove metals/heavy metals from water. The powdered shell may be explored for the treatment of toxic metals from industries, especially, electroplating industries, for the removal of metals from its outlet before entering the environment.

Conclusion

This communication presents a fast identification of $CaCO_3$ polymorphs present in shellfish by FTIR. Carbonate polymorphs present in clams, molluscs and gastropods are primarily aragonite in nature, whereas in oyster, it was found to be calcite. FTIR gave fast and cost-effective structural information for the identification of carbonate polymorphs present in shellfish. This identification was confirmed by XRD analysis of the dried and powdered exoskeleton shell. Further, the shell biomass was studied for metal adsorption using TDS and was found to be effective for the removal of metals thus; the biomass can be used for the treatment of water contaminated by metals/heavy metals. Therefore, this study enables an understanding of the structural arrangement of $CaCO_3$ in shellfish and the possible use of these waste materials for metal/heavy metal absorption from polluted water. It can be further explored as a remedial measure to treat the contaminated water released from electroplating industries before entering the environment.

Acknowledgements

We thank the Director, CSIR-NIO for the encouragement and Mr Girish Prabhu for the help in the collection of powder X-ray data. CSIR-NIO contribution No.: 6952.

Conflict of Interest

The authors have no conflict of interest to declare.

Ethical Statement

This work does not involve animal experiments which need ethical approval.

Author Contributions

KSS designed the experiments, analysed the data and wrote the manuscript. SGS performed the experiments and helped with data analysis.

References

1 Lowenstam H A, Minerals formed by organisms, *Science*, 211 (1981) 1126-1131.

2 Elner R, The mechanics of predation by the shore crab, *Carcinus maenas* (L.), on the edible mussel, *Mytilus edulis L.*, *Oecologia*, 36 (1978) 333-344.

3 Taylor J D & Layman M, The mechanical properties of bivalve (Mollusca) shell structures, *Palaeontology*, 15 (1972) 73-87.

4 Kunitake M E, Mangano L M, Peloquin J M, Baker S P & Estroff L A, Evaluation of strengthening mechanisms in

calcite single crystals from mollusc shells, *Acta Biomaterialia*, 9 (2012) 5353-5359.

5 Weiner S & Addadi L, Design strategies in mineralized biological materials, *J Mater Chem*, 7 (1997) 689-702.

6 Feng Q L, Cui F Z, Pu G, Wang R Z & Li H D, Crystal orientation, toughening mechanisms and a mimic of nacre, *Mat Sci Eng*, 11 (2000) 19-25.

7 Marshall D J, Santos J H, Leung K M Y & Chak W H, Correlations between gastropod shell dissolution and water chemical properties in a tropical estuary, *Mar Environ Res*, 66 (2008) 422-429.

8 Waldbusser G, Voigt E, Bergschneider H, Green M & Newell R, Biocalcification in the eastern oyster (*Crassostrea virginica*) in relation to long term trends in Chesapeake Bay pH, *Estuar Coasts*, 34 (2011) 221-231.

9 Lowenstam H A, Environmental relations of modification compositions of certain carbonate secreting marine invertebrates, *Proc Natl Acad Sci, USA*, 40 (1954) 39-48.

10 Dodd J R, The influence of salinity on mollusc shell mineralogy: A discussion, *J Geol*, 74 (1966) 85-89.

11 Watson S A, Peck L S, Tyler P A, Southgate P C, Tan K S, *et al.*, Marine invertebrate skeleton size varies with latitude, temperature and carbonate saturation: implications for global change and ocean acidification, *Global Change Biol*, 18 (2012) 3026-3038.

12 Yoshioka S & Kitano Y, Transformation of aragonite to calcite through heating, *Geochem J*, 19 (1985) 245-249.

13 Checa A G, Jiménez-López C, Rodríguez-Navarro A & Machado J P, Precipitation of aragonite by calcitic bivalves in Mg-enriched marine waters, *Mar Biol*, 150 (2007) 819-827.

14 Tatzber M, Stemmer M, Spiegel H, Katzlberger C, Haberhauer G, *et al.*, An alternative method to measure carbonate in soils by FT-IR spectroscopy, *Environ Chem Lett*, 5 (2007) 9-12.

15 Kontoyannis C G & Vagenas N V, Calcium Carbonate Phase Analysis Using XRD and FT–Raman Spectroscopy, *The Analyst*, 125 (2000) 251-255.

16 Ming N & Ratner B D, Differentiation of calcium carbonate polymorphs by surface analysis techniques - An XPS and TOF-SIMS study, *Surf Interface Anal*, 40 (2008) 1356-1361.

17 Anderson F A & Brecevic L, Infrared spectra amorphous and crystalline calcium carbonate, *Acta Chemica Scandinavica*, 45 (1991) 1018-1024.

18 Du Y, Lian F & Zhu L, Biosorption of divalent Pb, Cd and Zn on aragonite and calcite mollusk shells, *Environ Pollut*, 159 (2011) 1763-1768.

19 Prieto M, Cubillas P & Fernández-Gonzalez Á, Uptake of dissolved Cd by biogenic and abiogenic aragonite: A comparison with sorption onto calcite, *Geochim Cosmochim Acta*, 67 (2003) 3859-3869.

20 Asha K K, Anandan R, Mathew S & Lakshmanan P T, Biochemical profile of oyster *Crassostrea madrasensis* and its nutritional attributes, *Egypt J Aquat Res*, 40 (2014) 35-41.

21 Celik M Y, Çulha S T, Çulha M, Yildiz H, Acarli S, *et al.*, Comparative study on biochemical composition of some edible marine molluscs at Çanakkale coasts, Turkey, *Indian J Geo-Mar Sci*, 43 (2014) 601-606.

22 Molnár Á, The use of chitosan-based metal catalysts in organic transformations, *Coord Chem Rev*, 388 (2019) 126-171.

23 Croisier F & Jérôme C, Chitosan-based biomaterials for tissue engineering, *Eur Polym J*, 49 (2013) 780-792.

24 Anaya P, Cardenas G, Lavayen V, Garcı A & O'Dwyer C, Chitosan gel film bandages: correlating structure, composition, and antimicrobial properties, *J Appl Polym Sci*, 128 (2013) 3939-3948.

25 Wu Q, Chen J, Clark M & Yu Y, Adsorption of copper to different biogenic oyster shell structures, *Appl Surf Sci*, 311 (2014) 264-272.

26 Habte L, Shiferaw N, Khan M D, Thriveni T & Ahn J W, Sorption of Cd^{2+} and Pb^{2+} on aragonite synthesized from eggshell, *Sustainability*, 12 (2020) p. 1174.

27 Toffolo M B, Regev L, Dubernet S, Lefrais Y & Boaretto E, FTIR-based crystallinity assessment of aragonite-calcite mixtures in archaeological lime binders altered by Diagenesis, *Minerals*, 9 (2019) p. 121.

28 Balmain J, Hannoyer B & Lopez E, Fourier transform infrared spectroscopy (FTIR) and X-ray diffraction analyses of mineral and organic matrix during heating of mother of pearl (Nacre) from the shell of the mollusc *Pinctada maxima*, *J Biomed Mater Res*, 48 (1999) 749-754.

29 Korash C A, Mechanical properties of a nanostructure poly (KAMPS)/ aragonite composite in mechanical of biological system and materials, *Conference proceeding of the Society for Experimental Mechanic Series*, 2 (2011) 131-136.

30 Ni M & Ratner B D, Differentiation of calcium carbonate polymorphs by surface analysis techniques-An XPS and TOF-SIMS study, *Surf Interface Anal*, 40 (2008) 1356-1361.

31 Nakamoto K, Fujita J, Tanaka S & Kobayashi M, Infrared spectra of metallic complexes. IV. Comparison of the infrared spectra of unidentate and bidentate metallic complexes, *J Am Chem Soc*, 79 (1957) 4904-4908.

32 Weir C E & Lippincott E R, Infrared studies of aragonite, calcite, and vaterite type structures in the borates, carbonates, and nitrates, *J Res Natl Bur Stand A Phys Chem*, 65A (1961) 173-180.

33 Sato M & Matsuda S, Structure of vaterite and infra-red spectra, *Z Kristallogr*, 129 (1969) 405-410.

34 Donoghue M, Hepburn P H & Ross S D, Factors affecting the infrared spectra of planar anions with D3h, symmetry-V: The origin of the splitting of the out-of-plane bending mode in carbonates and nitrates, *Spectrochim Acta Part A: Mol Spect*, 27 (1971) 1065-1072.