



Research Article

Properties of N, acetylamide-rich biopolymers from five different aquatic invertebrates

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ABSTRACT

There have been huge demands for aquaculture products over the years owing to continuous population growth the globe consistently witnesses. Marine invertebrates such as the molluscs and crustaceans are affirmed to be good source of protein. The shells of these animals however are often discarded after the edible parts are consumed; this ends up polluting the environment. A useful material that can be sourced from these shells is a natural polymer – chitin. In this study, chitin was chemically extracted from ground snail, periwinkle, crab, shrimp and prawn shells under similar isolation conditions using 1 M hydrochloric acid and sodium hydroxide. This was done to investigate the influence of each shell on the ease of chitin isolation and eventually, its properties. It was observed that property of chitin depended on features of each shell. The harder the shell, the longer it was to achieve complete demineralization as more acid volumes were consumed. A maximum volume of 2490 ml was needed for total demineralization of 150 g periwinkle while the least (1498 ml) was required for shrimp shell of the same quantity. This implied that snail and periwinkle shells contain more inorganic minerals other than the dominant calcium carbonate. Degree of acetylation, calculated from Energy Dispersion Spectroscopy results, was the maximum for shrimp chitin (79.9 %) while the least (61.6 %) was calculated for periwinkle chitin. Thermogravimetry Analysis showed that 47.8, 15, 60.8, 78.4 and 63.7 % chitin was yielded from periwinkle, crab, prawn and shrimp shells respectively. The highest value of degree of acetylation possessed by shrimp chitin was responsible for its superlative thermal stability (324.6 kJ). Average intra and intermolecular OH bonds in shrimp chitin was calculated to be 5.461 kCal, which was the highest magnitude; 4.642, 4.543, 4.696 and 4.952 kCal were calculated for snail, periwinkle, crab and prawn chitin respectively.

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INTRODUCTION

Majority of the wastes in the food industry comprise high amount of biochemical oxygen demand (BOD)

components; these wastes are recoverable as they are organic in nature containing vitamins, carbohydrates, fats and proteins [1]. Continuous demands in sea foods' edible

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products for consumption have greatly influenced activities in aquaculture globally over the years owing to speedy population growth. In the tropical and subtropical oceans (Pacific, Atlantic and Indian), lobsters, prawns, crabs and shrimps have been of great commercial importance; their shells have been a major source of chitin. Prawns, although look like shrimps, they are usually bigger in terms of size while both crustaceans find their habitats in salt and fresh water [2,3]. According to Soundhar and Kandasamy [4], the crab shell is the second most common natural composite in the world; it prevents the entire body from damage through external loads, harsh temperatures and chemicals. This can also be said about shells of molluscs such as snail and periwinkle, which play key role in protecting their living tissues. Farming of molluscs in recent times has been proven to be a sustainable food source and it is affirmed to be a potential operation for food security in the future [5]. The shells which were earlier reported to pollute the environment have been a huge raw material for cement [6], hydroxyapatite [7] and farming [5] owing to the large content of calcium carbonate /calcite/lime stone (CaCO_3) present in them. In many countries, snails are regarded as an epicurean delicacy and it is being assessed as a good source of protein [8]. The shell of snail has been proven to contain 95 wt. % CaCO_3 and 5 wt. % organic constituents [9] but earlier studies have reported that the 95 wt. % comprises P, Mn, Fe, Cu, Zn, Na and K – containing minerals [10]. Potorti et al. [11] reported that snail shell wastes are a good source of chitin, chitosan and CaCO_3 . Same have been affirmed about periwinkle shell – it contains CaCO_3 as a major inorganic mineral [12,13]. In the findings of Aranaz et al. [14], it was confirmed that crustacean exoskeletons are made of inorganic mineral - CaCO_3 , (30–50 %), protein (30–40 %) and chitin (20–30 %). Chitin is a natural polymer second to cellulose in terms of abundance and it has also been reported to exist in the shells of molluscs [15]. It can thus be said that the shells of molluscs and crustaceans are composites comprising CaCO_3 , protein and chitin. This natural polymer is a polysaccharide whose chains are strongly gripped by hydrogen bonds (Figure 1a). This tight network is linked by the strong carbonyl ($\text{C}=\text{O}$) and amine ($\text{H}-\text{N}$) groups, which are responsible for its thermal, mechanical and chemical properties. The combination of these groups makes up the amide (CONH_2) group that basically defines the structure of chitin. This natural polymer has found its applications in medicine [16], food packaging [17], water treatment [18], adhesives and tissue engineering [19]. Isolating chitin from these shells is often done conventionally using acid and alkali solutions of different concentrations [20,21]. The former eliminates CaCO_3 (demineralization) while the latter separates protein from the organic matrix (deproteinization) [20,22–24], leaving chitin to be directly used for its desired applications or for further processing such as depigmentation [22, 25] and deacetylation to chitosan (a derivative of chitin) [19]. Here, $\text{C}=\text{O}$ is eliminated leaving $\text{H}-\text{N}$ in its structure (Fig. 1b) [26,27].

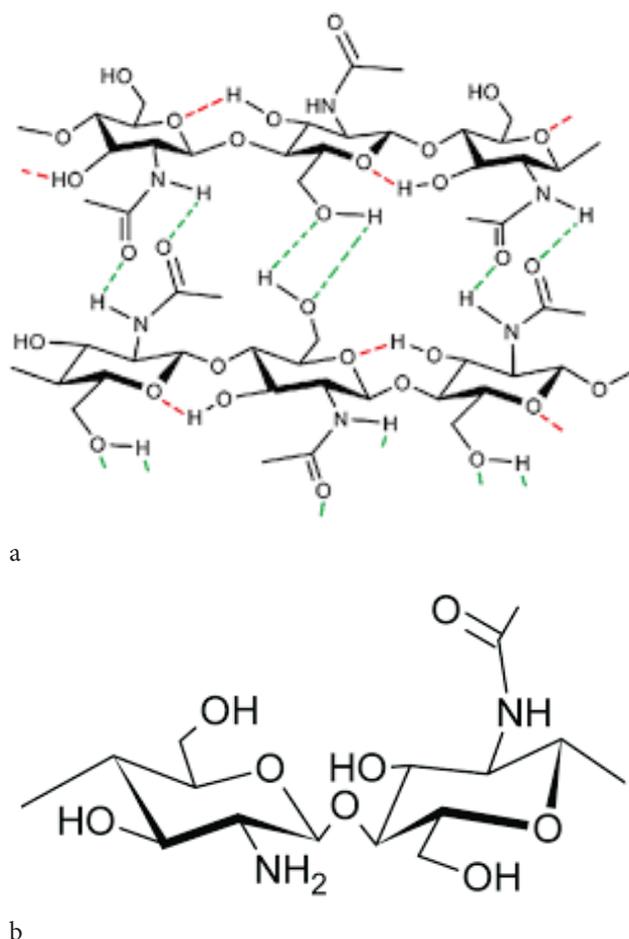


Figure 1. (a) Chitin structure and its layers connected by inter and intra molecular hydrogen bonds (b) structure of chitosan.

In some cases, deproteinization may precede demineralization during the process of chitin isolation. This was executed by Adekanmi et al. [28] where dry snail shell particles were first deproteinized using 2 % (wt. /v) NaOH at 90 °C for 2 h. Mineral contents removal was later achieved by reacting deproteinized shells with 2.5 % (v/v) HCl at room temperature for 6 h. This method was contrary to that practised by Rajathy et al. [29] who first demineralized telescope snail shells with 5 % (v/v) HCl at room temperature for 48 h. For the same period, protein was later removed using 3 % (wt. /v) NaOH at 70 °C. Within 1 h, Ayorinde and Edema [30] refluxed ground periwinkle shells in 1 M HCl at 100 °C for demineralization. This was followed by deproteinization where 1 M NaOH was used at 80 °C for 1 h. The first stage of chitin production from shrimp shell as engaged by Fonseca et al. [31] was depigmentation which entailed the use of 80 % (v/v) ethanol/water solution. Shrimp shells have been confirmed to contain pigments such as carotenoproteins and carotenoid [32], which is believed to influence chitin's colour and its properties. At room temperature and 50 min steady agitation, the researchers [31] demineralized shrimp

shells with 5.2 % (v/v) HCl, followed by reaction with 4 % (wt./v) NaOH for protein separation. A reagent commonly used for depigmentation/ decolouration is hydrogen peroxide (H₂O₂) which has been used by Zhang et al. [33]. Unlike Fonseca et al. [31], Zhang et al. [33] performed depigmentation after ground shrimp shells with Natural deep eutectic solvent (NADES). The solvent comprised choline chloride and organic acids; it was used as an alternative to demineralization and deproteinization process. Chitin has been sourced from ground crab shells following three stages – deproteinization, depigmentation and demineralization [34]. Protein was removed from the shells using 5 % (wt./v) NaOH for 10 h at 80 °C. Soaking the protein-free shells in acetone for 24 h removed the pigments while treatment with 1 % (v/v) HCl for 24 h was carried out to eliminate CaCO₃. Mohan et al. [35] successfully isolated chitin from crab shell by following the sequence, demineralization (2.5 M HCl at 60 °C and 2.5 h), deproteinization (3 M NaOH at 80 °C and 2 h) and depigmentation (chloroform/methanol/distilled water solution). In the works of Novikov et al. [36], chitin was processed from king crab shell without depigmentation. The first step involved reaction with 4 % (wt./v) NaOH at 95 °C for deproteinization while demineralization was achieved at room temperature with 3.6 % (v/v) HCl for 30 min. Sreeja et al. [37] also sourced chitin from powdered crab shells without depigmentation. Unlike Novikov et al. [36], demineralization was performed first by treating the powder with 2 N HCl for 24 h; with the same period, 2 N NaOH at 80 °C dissolved the protein present.

Studies however have shown that irrespective of the processing sequence followed, chitin can be successfully extracted from shells of crustaceans and molluscs, [15,20,22], where different acid and alkali solutions (with varying concentrations and temperatures) have been employed. The diverse conditions of chitin isolation from these different sources have made it difficult to decipher how the nature of each shell influences the ease of chitin isolation with its properties. This can only be possible if shells of crustaceans and molluscs are chemically treated under similar conditions. In this study, we have decided to chemically isolate chitin from shells of five different marine invertebrates – snail, periwinkle, crab, shrimp and prawn under same experimental conditions. With this, the role of each shell nature on the ease of chitin isolation is deciphered; properties of each isolated chitin are investigated and comparisons are made.

MATERIALS AND METHODS

Isolation of Chitin From Different Shells

Snail, periwinkle, crab, prawn and shrimp shells were thoroughly washed with distilled water to remove other tissues after edible parts were removed. Shells of each invertebrate were sun-dried, ground and milled to 250 µm particles. In order to remove the inorganic mineral(s)

present in each shell, volumes of 1 M HCl at room temperature were made to react with each shell (150 g) until the emission of carbon (IV) oxide, CO₂ ceased. The mineral-free particles (from each source) were separately washed with distilled water and oven-dried at 75 °C for 5 h after filtering. Protein removal (deproteinization) was achieved by refluxing mineral-free particles with 1 M NaOH at 100 °C for 1 h. At the end of this process, particles were washed, filtered and completely dried in an oven as earlier done. Processed chitin samples were stored in moisture-free covered containers prior to characterizations. For clarity purpose, the following designations, S, P, C, Pr and Sh are used to represent snail, periwinkle, crab, prawn and shrimp respectively in this study.

CHARACTERIZATIONS

Elemental Constituents

Constituents of shells with their corresponding chitin produce were studied with the use of an energy dispersion spectrometer (EDS). This device determined the extent of isolation by detecting elements present before and after chemical treatments.

Thermogravimetric Analysis (TGA)

Temperatures at which chitin began and completed its decomposition including the dominant mineral contents were measured using PerkinElmer TGA 4000 equipment. Each powdered sample of 2 mg (from both shells and chitin) was heated from 0 - 750 °C at a heating rate of 10 °C/min.

Fourier Transform Infrared Spectroscopy (FTIR)

Functional groups present in both shells and isolated chitin was detected with a spectrometer (PerkinElmer Spectrum Two) in a transmittance mode between 500–4000 cm⁻¹ and 4 cm⁻¹ resolution.

RESULTS AND DISCUSSION

Volume of Acid Consumed

The total volume of HCl used to completely demineralize each shell is given in Table 1. Periwinkle and snail shells require much of the acid compared to other shells. It can be concluded here that the harder the shell, the more volume of HCl that will be required for complete demineralization. It may also be inferred that more minerals are present in the shells of periwinkle and snail which may be strongly bonded to chitin embedded in them. Shrimp and prawn shells, whose HCl demineralization volume is in the difference of 0.8 %, can be easily demineralized compared to crab shells. It should be noted here that the volume of HCl used just indicates the ease of mineral removal from each shell. Values may differ for other experiments based on parameters such as type and concentration of acid used, particle

Table 1. Total volume of HCl consumed

Shell	HCl volume (ml)
S	2476
P	2490
C	1796
Pr	1510
Sh	1498

size and specie of shells, temperature of demineralization, etc. This study just establishes that under same conditions, chitin can be easily extracted from prawn and shrimp shells rather than from periwinkle and snail shells.

Elemental Constituents

The EDS results show that periwinkle shell contains the highest content of silica (SiO_2) as indicated by Si (14.5 %) followed by snail shell, containing 12.3 % (see Table 2). This (coupled with the presence of CaCO_3) may be responsible for the high volumes of HCl required for their complete demineralization. All virgin shells contain calcium (Ca), which maintains the highest amount for each shell, indicating that CaCO_3 is a dominating mineral in the exoskeletons of these marine invertebrates. Demineralization and deproteinization of these shells have imparted significant reductions in these minerals with increase in carbon (C) contents implying chitin formation. In this study, the acetylation degree (DA), which is a measure of acetyl group ($\text{RC}=\text{O}$) present in the whole chitin chain is calculated using C/N ratio in equation (1) as employed by Abdou et al. [38].

$$D.A = \frac{C/N - 5.14}{1.72} \times 100 \quad (1)$$

Carbon and nitrogen are represented by C and N respectively. The ratio is got from the elemental content as determined via EDS. Calculation shows that DA of 65.3, 61.6, 72.2, 79.9 and 79.8 % is possessed by snail, periwinkle, crab, shrimp and prawn chitin. This implies that shrimp and prawn chitins have the highest $\text{C}=\text{O}$ amount (approximately 80 %) in their chains. Young's Modulus of chitin has been reported to be directly linked with its DA [39]; this implies that the higher chitin's DA, the stiffer it becomes. Also, when chitin has more of $\text{C}=\text{O}$ units in its structure, it becomes less prone to aqueous solution attacks – it becomes more hydrophobic [40]. The enhanced elastic modulus qualifies chitin to be used as a temporary supporting device in tissue engineering [41]. Balancing high chitin's stiffness with improved hydrophilicity can be achieved by controlling its $\text{C}=\text{O}$ units with hydrophilic polymers such as cellulose chitosan and polyvinyl alcohol (PVA) [42,43].

Thermal Stability and Yield of Chitin

Thermogravimetric analysis results of each shell and its corresponding isolated chitin are illustrated in Tables 3 and 4. Each stage of decomposition depicts the existence of moisture, chitin or CaCO_3 . Each virgin shell is characterized with three decomposition stages while each isolated chitin TGA possesses two. Decomposition stage between 90 – 110 °C represents the elimination of moisture content existing in each sample and this ranges between 0.5 – 10.1 wt. %. The TGA has been able to identify the occurrence of CaCO_3 in each untreated shell which justifies the prevalence of this mineral. Temperatures of commencement (T_{onset}) and end of decomposition (T_{finish}) of chitin and CaCO_3 with their contents are also illustrated in Table 3. High amount of residue present in the TGA of untreated snail and periwinkle shells imply the existence of minerals other than SiO_2 and CaCO_3 that is not identified via this analysis owing to the temperature range employed. Treatment of each shell with acid and alkali solutions further increases chitin content with reduced residue which shows that there exist some compounds present (in the residue) which is not identified via TGA. The incomplete (majorly from snail and periwinkle shells) and/or non-isolation of other unidentified minerals existing in these shells may be responsible for incomplete chitin isolation. The highest content of chitin can be realized from shrimp shell; this is followed by prawn shell. This study shows that the harder the shell, the lower the amount of chitin that can be yielded (Table 4). Thermal stability of chitin explains the energy required to break its glycosidic linkage. Nam et al. [44] explained that the N- acetyl groups ($\text{C}=\text{O}$) in chitin plays a key role in its thermal stability. This implies that chitin with a high DA (more of $\text{C}=\text{O}$ units) is expected to be stable. In this study, thermal stability of each isolated chitin is quantified from activation energy calculations from the TGA results according to equation (2) [45].

$$\ln(-\ln(1-X)) = -Ea/RT + \text{Const.} \quad (2)$$

The absolute decomposition temperature is designated as T (K) while the activation energy of the decomposition is Ea (kJ/mol); R has a value of 8.314 J/mol·K (universal gas constant). Decomposition degree, X is obtained using equation (3):

$$X = [(W_o - W_i) / (W_o - W_f)] \quad (3)$$

Shrimp chitin possesses the highest thermal stability while periwinkle chitin is the least thermally stable (Table 4). This further justifies the findings of Nam et al. [44] as increasing trend of DA for each isolated chitin sample corresponds with the trend of Ea. In addition, the existence of impurities could be responsible for chitin's thermal stability; the more the impurities in the shell, the lower the thermal stability of isolated chitin. All chitin samples have

Table 2. Elemental composition results of shell and chitin from snail, periwinkle, crab, shrimp and prawn via EDS

Elements (%)	S. shell	S. chitin	P. shell	P. chitin	C. shell	C. chitin	Sh. shell	Sh. chitin	Pr. shell	Pr. chitin
O	50.1	69.8	44.3	69	71.5	74.6	77.2	73.2	75.1	70.2
Si	12.3	1.2	14.5	1.2	0.8	--	0.4	--	--	--
C	3.7	23.8	8.1	24.8	10.2	21.7	5.9	22.8	7.7	25.4
N	5.1	3.8	4.7	4	5.4	3.4	6.6	3.5	6.1	3.9
Ca	28.8	1.4	28.4	1	10.8	0.3	9.9	0.5	11.1	0.5
Mg	--	--	--	--	1.3	--	--	--	--	--

Table 3. TGA results for untreated snail, periwinkle, crab, shrimp and prawn shells

Shell	Chitin			CaCO ₃			Residue (%)
	T _{onset} (°C)	T _{finish} (°C)	Amount (%)	T _{onset} (°C)	T _{finish} (°C)	Amount (%)	
S	234	490	2.9	636	782	41.4	54.5
P	232	490	2.7	632	748	20.3	76.5
C	285	474	11.1	640	721	28.7	54.7
Sh	282	407	37.2	646	712	15.2	38.9
Pr	275	407	47.2	669	735	9.4	33.3

Table 4. TGA results for isolated chitin from snail, periwinkle, crab, shrimp and prawn shells

Shell source	T _{onset} (°C)	T _{finish} (°C)	Amount (%)	Residue (%)	Ea (kJ)
S	260	399	47.8	45.7	59.0
P	277	419	15	81.7	57.4
C	290	420	60.8	29.1	99.8
Sh	298	427	78.4	13.9	324.6
Pr	283	407	63.7	30.7	270.2

high T_{onset} (beyond 250 °C) and this implies that they can find a potential use within this temperature range in areas such as food packaging and medical devices including dialyzers, sterilizing boxes, etc. Majority of the polymers used for these applications are fossil fuel based.

Functional Groups and OH Bonding

The functional groups present in each shell and their associated isolated chitin is presented in Figure 2a and b. Each shell spectrum justifies the existence of CaCO₃ as a dominant inorganic mineral, which is more on snail and periwinkle shells. This mineral is formed on 866 and 668 cm⁻¹ in both shells (Figure 2a) while it forms as one peak in crab, shrimp and prawn shells Major conventional chitin functional groups (Amides I, III and III) are formed after isolation (Figure 2b) with their OH regions stretching wider. Chitin spectra are similar to that investigated by Algethami et al. [34]. Tables 5 and 6 identify the functional groups in untreated shells and isolated chitin respectively.

The intra and intermolecular hydrogen bonds in each chitin sample was obtained from FTIR fitting via Gaussian deconvolution between 3600 and 3000 cm⁻¹ (Figure 3a - e). The intra molecular bonds (OH(6)...OC and OH(3)...O(5)) exist between 3461- 3521 cm⁻¹ and 3269 - 3497 cm⁻¹. In addition, between 3257 - 3281 cm⁻¹ lie intermolecular CO...HN bond is formed; another intermolecular bond (OH...OC) exists between 3052 and 3098 cm⁻¹. The hydrogen bond energy E_H for each chitin sample between 3600 and 3000 cm⁻¹ was determined by applying equation (4) [46]:

$$E_H (kCal) = \frac{1}{K} x \frac{V_o - V}{V_o} \quad (4)$$

K is a constant (1.68 × 10⁻² kcal⁻¹); V_o takes the value of 3600 cm⁻¹ (standard wavenumber of OH) and V is the corresponding wavenumber of bonded OH

The hydrogen bond energy E_H magnitude for each chitin variant increases as the wavenumber is towards 3000

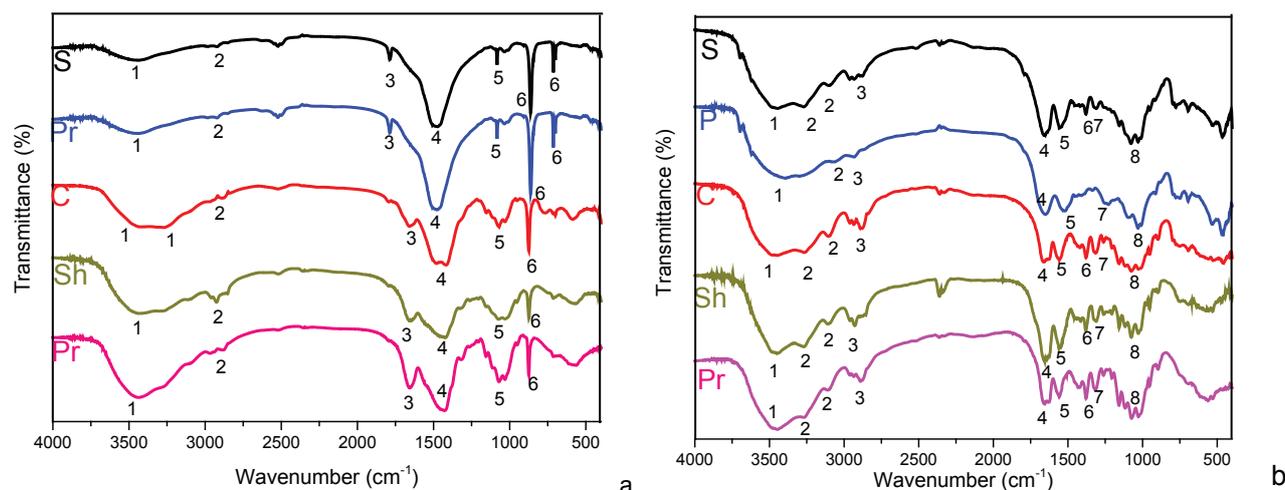


Figure 2. FTIR spectrum of snail, periwinkle, crab, shrimp and prawn (a) shells and (b) chitin.

Table 5. Functional groups of untreated snail, periwinkle, crab, shrimp and prawn shells

Functional groups	Wavenumber (cm ⁻¹)				
	S	P	C	Sh	Pr
1 OH stretching	3443	3456	3456, 3266	3449	3436
2 CH ₃ (symmetric) and CH ₂ (asymmetric) stretching	2903	2903	2910	2942	2872
3 C=O (amide I)	1768	1799	1659	1653	1672
4 CH ₂ (bending) and CH ₃ (deformation)	1488	1481	1481	1437	11418
5 C-O-C	1082	1082	1075	1056	1069
6 CaCO ₃	866, 668	866, 668	877	859	859

Table 6. Functional groups of isolated chitin from snail, periwinkle, crab, shrimp and prawn shells

Functional groups	Wavenumber (cm ⁻¹)				
	S	P	C	Sh	Pr
1 OH stretching	3494	3374	3469	3469	3456
2 NH stretching	3240, 3094	3008	3253, 3008	3259, 3101	3240, 3101
3 CH ₃ (symmetric) and CH ₂ (asymmetric) stretching	2922	2903	2910	2916	2903
4 C=O (amide I)	1666	1659	1659	1653	1659
5 NH and CN (amide II)	1520	1520	1545	1538	1551
6 CH ₂ wagging (amide III)	1355	--	1367	1367	1361
7 Asymmetric bridge oxygen stretching	1285	1253	1298	1285	1291
8 C-O-C	1049	1031	1043	1043	1049

cm⁻¹ implying that each intermolecular bond in chitin is stronger than intra molecular bond (Table 7). The average E_H is of the highest magnitude in shrimp chitin while periwinkle chitin maintains the least. Results from this study show that increasing contents of CaCO₃ (with other

inorganic minerals) present in the shell of each marine invertebrate lowers the average E_H of each isolated chitin. This could be as a result of more volumes of HCl required to demineralize harder shells.

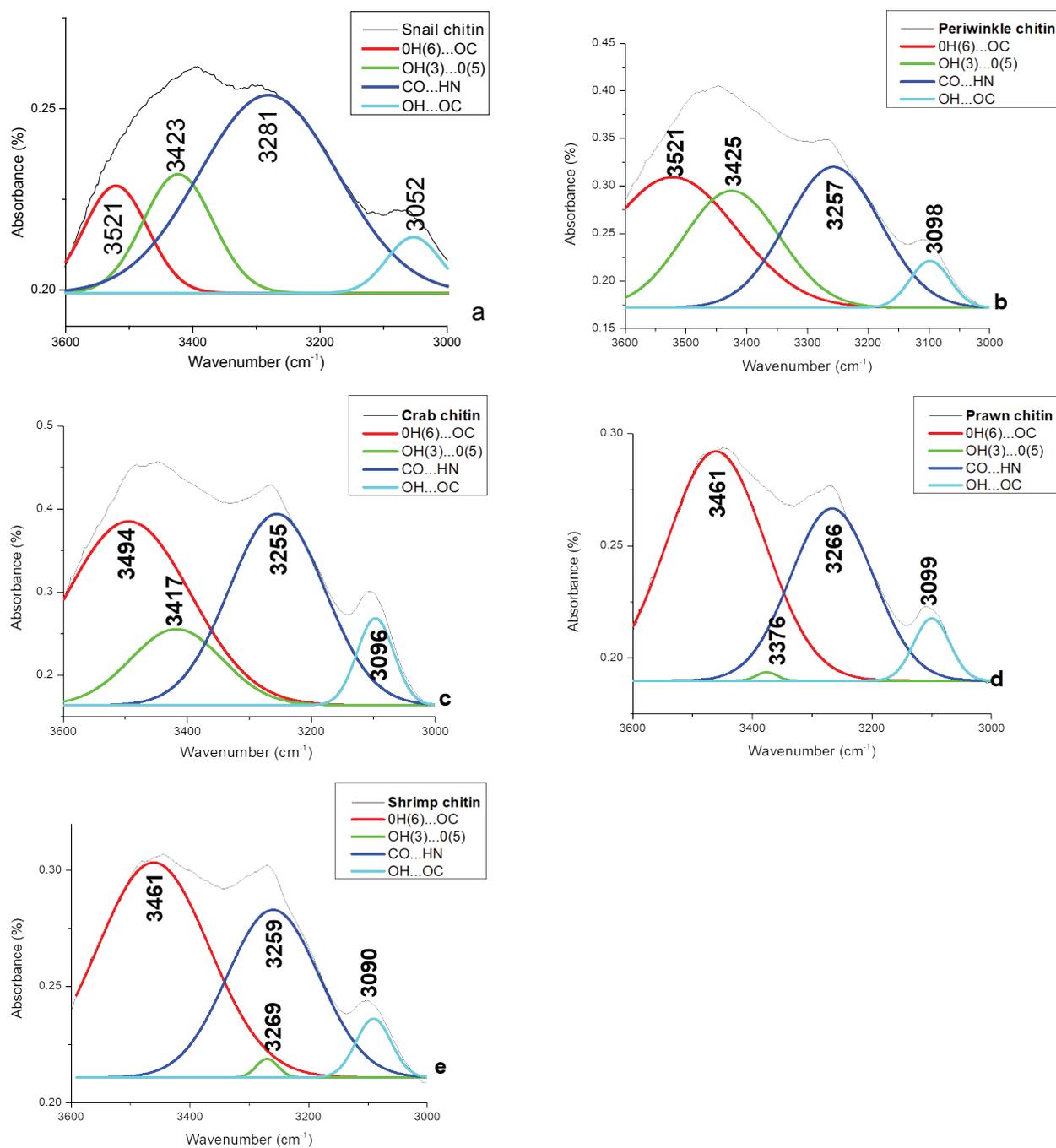


Figure 3. Spectra fitting of OH of (a) snail (b) periwinkle (c) crab (d) prawn and (e) shrimp chitin.

Table 7. Hydrogen bond energies of isolated chitin from snail, periwinkle, crab, prawn and shrimp shells

H-Bond energy, E_H (kCal)					Average E_H (kCal)
Chitin	OH(6)...OC	OH(3)...O(5)	CO...HN	OH...OC	
S	1.306	2.927	5.274	9.061	4.642
P	1.306	2.894	5.671	8.3	4.543
C	1.753	2.993	5.704	8.333	4.696
Pr	2.298	5.473	5.638	8.433	4.952
Sh	2.298	3.704	5.522	8.284	5.461

CONCLUSION

Chitin has been successfully isolated chemically from shells of snail, periwinkle, crab, shrimp and prawn using 1 M hydrochloric acid and sodium hydroxide. Comparing these shells (and their corresponding isolated chitin) with one another, this study has shown that under similar extraction conditions, it is difficult to isolate chitin from snail and periwinkle shells compared to other shells (crab, shrimp and prawn) as evidenced in the total volume of acid solution consumed. This could be attributed to a strong calcium carbonate – chitin bond and/or presence of other inorganic minerals existing in these molluscs' shells. The reduced inorganic minerals and weak mineral-polymer bond in shrimp and prawn shells culminates in a comparable repeating C=O units of 79.9 and 79.8 % respectively while crab chitin possesses 72.2 %; this is greater than that of snail (65.3 %) and periwinkle (61.6 %) chitins. It is expected that chitin with the highest C=O units (by virtue of acetylation degree value) should display the maximum mechanical properties when in use. This is also related to the average hydrogen bond energy which ranges in the order: shrimp chitin > prawn chitin > crab chitin > snail chitin > periwinkle chitin. The intra and intermolecular bonds (coupled with C=O repeating units) is also responsible for chitin's thermal stability in the same order as earlier stated. Considering similar extracting conditions as devised in this study, maximum amount of chitin can be realized from shrimp shell and the least, from shell of periwinkle. Thermal result from thermogravimetric analysis shows that all virgin shells with their isolated chitins contain some residues which is very high for periwinkle chitin. The decomposition temperature range (0 – 750 °C) employed for the analysis was not sufficient to detect other inorganic materials present. To achieve improved chitin yield from these shells (most especially snail and periwinkle), more studies need to be carried out to identify these minerals and effective methods to isolate them needs to be devised. We are undertaking further studies to achieve this.

AUTHOR CONTRIBUTIONS

GBENEBOR O.P.: conceptualization, investigation, methodology, data analysis, writing an original draft, and reviewing; POPOOLA A.P.I.: investigation, methodology, editing and funding acquisition. Authors have read and agreed to the published version of the manuscript.

DATA AVAILABILITY STATEMENT

The authors confirm that the data that supports the findings of this study are available within the article. Raw data that support the finding of this study are available from the corresponding author, upon reasonable request.

CONFLICT OF INTEREST

The author declared no potential conflicts of interest with respect to the research, authorship, and/or publication of this article.

ETHICS

There are no ethical issues with the publication of this manuscript.

STATEMENT ON THE USE OF ARTIFICIAL INTELLIGENCE

Artificial intelligence was not used in the preparation of the article.

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