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A sustainable approach to water quality improvement of aquatic ecosystems using low-cost chitosan from discarded crustacean exoskeletons as an adsorbent for heavy metal removal

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Abstract

Heavy metals are considered hazardous chemicals in the environment and, under certain conditions, become toxic to plants, animals and humans. Heavy metal contamination in water resources poses a significant environmental challenge. Chitosan, a biopolymer derived from chitin, has emerged as a promising adsorbent for heavy metal removal. This review firstly focuses on the extraction of chitosan from discarded crustacean exoskeletons and secondly on the application of Isothermal Titration Calorimetry (ITC) to investigate the thermodynamics of metal ion binding to chitosan. ITC provides crucial insights into the binding mechanisms, including the number of binding sites, binding affinities, and the driving forces behind the interactions. Additionally, this review synthesizes findings from studies employing ITC to understand the potential of chitosan for heavy metal remediation. Magnesium (II) is found to exhibit the strongest affinity toward chitosan. Zinc (II) is the next strongest cation, while calcium (II) is found to show the weakest measurable affinity for chitosan chelation. Binding of all these cations to chitosan takes place with negative free energy values. Based on the results, ITC method clearly demonstrates that chitosan is a versatile bio polymer that can effectively bind the three metals studied minimizing the impact of these elements on the aquatic ecosystem.

Keywords: Chitosan; Isothermal Titration Calorimetry; Metal Binding; Crustacean; Water Quality

1. Introduction

Crustaceans, belonging to the Decapoda order, include prawns, shrimps, lobsters, crayfish and crabs, are regarded as an enriched source of many bioactive substances such as chitin, chitosan, glucosamine and astaxanthin. Melicerthus kerathurus known as karamote prawn is one of the above group. The karamote prawn Melicertus kerathurus (FORSK L, 1775) is a native species in the Mediterranean Sea and the East Atlantic [1,2].

Chitin, a carbohydrate bio-polymer, is a linear polysaccharide consisting of N-acetylglucosamine (GlcNAc) residues only, but chitosans are linear co-polymers of GlcNAc and glucosamine (GlcN) residues [3,4]. Due to their useful biological properties (bio-compatibility, biodegradability, antimicrobial activity) and chemical modification potentials because of their reactive functional groups (-OH, -NH2, and -COOH) both have been used in a wide range of fields including biomedical, food production, and wastewater treatment fields. Chitin can be obtained from the cell wall of fungi, the exoskeleton of arthropods, the shells of mollusks and the beaks of cephalopods including cuttlefish, octopuses and squids. Chitin is presented mainly in three allomorphs: α-chitin, with antiparallel chains, is the most abundant and it is isolated from the exoskeleton of crustaceans, particularly from shrimps and crabs; β-chitin, with parallel chains, is

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presented in the cell walls of diatoms and in the skeletal structures of cephalopods and commonly extracted from squid pens; γ-chitin is presented in fungi and yeast, which is a combination of the α and β allomorphs. Chitosan, a naturally abundant bio-polymer, is generally prepared by the deacetylation of chitin with alkali [3, 5,6,7,8,9]

Heavy metal pollution is a severe environmental problem due to the toxicity and persistence of these pollutants. These persistent and toxic substances can enter food chains through bioaccumulation, leading to serious health problems and damaging ecosystems [10,11,12]. Chitosan has gained attention as a potential adsorbent for heavy metal removal due to its biocompatibility, biodegradability and ability to bind metal ions [10,11,13,14,15]. The ITC (Isothermal Titration Calorimetry) instrument is a heat-flux calorimeter operating according to the dynamic power compensation principle. It measures accurately the amount of power required to maintain a constant temperature difference between the sample and the reference cell; directly measures the heat changes associated with binding affinities of molecular associations providing a comprehensive thermodynamic characterization of these interactions. Initially, the feedback system continuously applies a small power to the sample cell, which determines the baseline level. Each injection of the syringe solution (ligand) triggers the binding reaction and depending on the binding affinity and the concentration of reactants (chitosan and metal) in the cell, a certain amount of macromolecule/ligand (ML) complex is formed. The formation of complex (chitosan and metal) is accompanied by the release or the absorption of heat (exothermic or endothermic reaction) that causes a difference in temperature between the two cells. Then, the feedback system either lowers or raises the thermal power applied to compensate such temperature unbalance. After each injection, the system reaches equilibrium and the temperature balance is restored [16-21]. ITC has the ability to determine precisely the Gibbs energy, enthalpy, entropy and heat capacity changes associated with binding event [16,18,20].

This review focuses on the application of ITC in studying divalent metal ions binding to chitosan. Understanding the thermodynamics of the interaction between chitosan and metal ions is essential for optimizing adsorption processes.

2. Materials and methods

The experimental fishery of the shrimps was carried out three times from October 2023 to January 2024 in Kastos channel (Figure 1), along the Western Greek coast of the Ionian Sea, near the Kalamos Island (Latitude: 38°33'59.99"N, Longitude: 20°54'59.99"E).

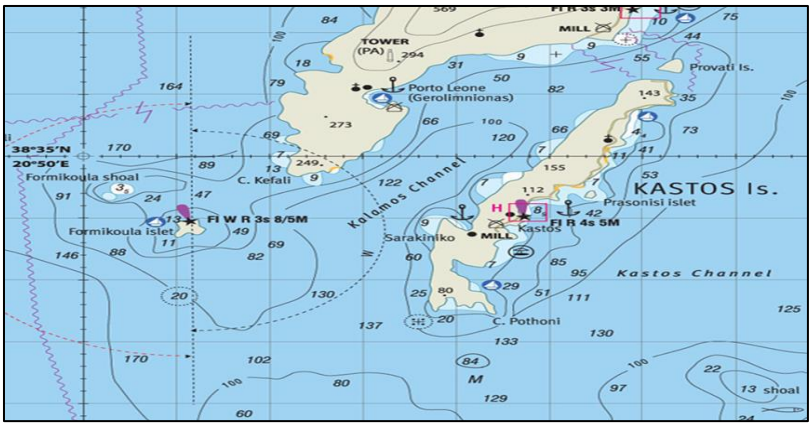

Figure 1 Map of the study area in Kastos channel along the Western Greek coast

All the chemicals and solvents (sodium hydroxide, hydrochloric acid, acetone, hexane, ethanol, acetic acid) used were purchased from Sigma-Aldrich at the analytical grade level of purity available and used as received. For the preparation of solutions, double distilled water was used.

The sample shells were removed from the animal and secondly the specimens were packed in polyethylene bags, placed on ice, transported to the laboratory and were stored in a freezer at -20 °C until further use. Before grinding, the biggest parts of shell samples were crushed and divided in smaller. Drying of samples was obtained by heating in a drying oven (model R. Espinar, S.L.) at 95 °C until constant weight was obtained between two sequential measurements (3 h). Drying samples were grinded in a mill (System POLYMIX® PX-MFC 90 D) into smaller particles using sieve with 2 mm wide openings. Flow diagram of extraction of chitosan from shrimp shells is observed in Figure 2 while Figure 3 shows photo of shrimp shells after treatment and chemical process (demineralization, deproteination, deacetylation).

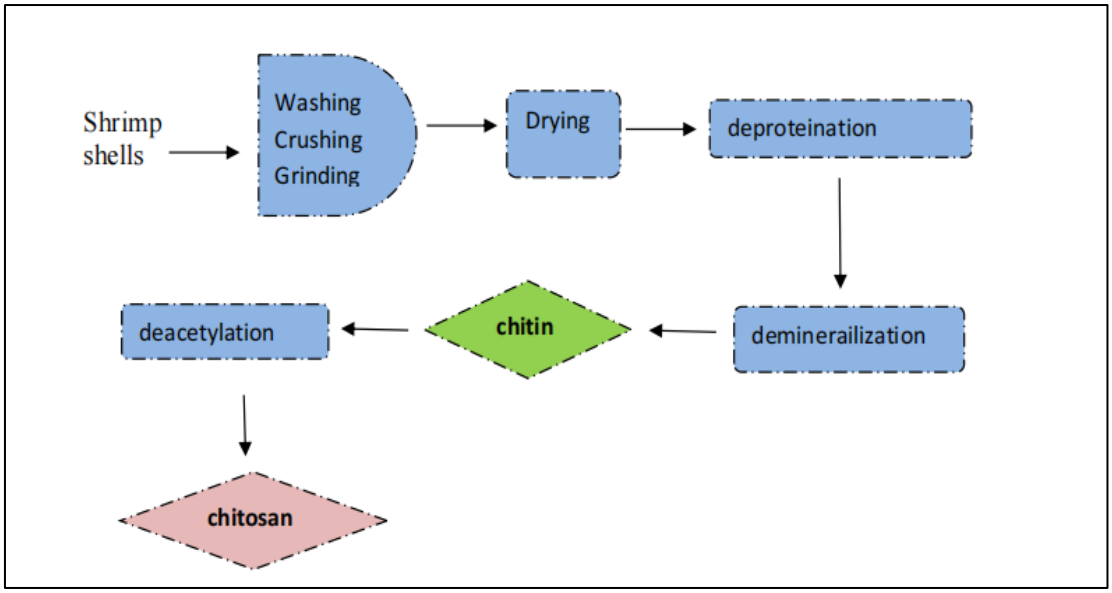

Figure 2 Flow diagram of extraction of chitosan from shrimp shells

2.1. Extraction of chitin by chemical method

In the first step deproteinization (Dp) occured. Dry samples of raw shrimp waste were treated with diluted solution of NaOH at different solid to solvent ratio (w/v), with constant stirring for twelve hours at different temperatures, with pH ranged from 11-13. After that, the solution was filtered and the samples were washed with distilled water and ethanol to neutrality.

In the second step demineralization (Dm) followed. Samples from deproteination process were treated with diluted solution of HCl at different solid to solvent ratio (w/v), with constant stirring for twelve hours with pH value ranged pH 1.0-2.5 at room temperature. After that, the solution was filtered and the samples were washed with distilled water to remove acid and calcium chloride. The samples were dried until constant weight was obtained. The dried sample is now known as chitin [1,2,22].

2.2. Chitosan production

The deacetylation (Da) process was conducted by soaking dried chitin prepared from demineralization in a concentrated solution of NaOH or (w/v) KOH with constant stirring for one day at room temperature. After that, the product is known as chitosan. Chitosan was washed with tap water until neutral (pH 6.5-8.0) and dried until constant weight was obtained [1,22,23,24,25].

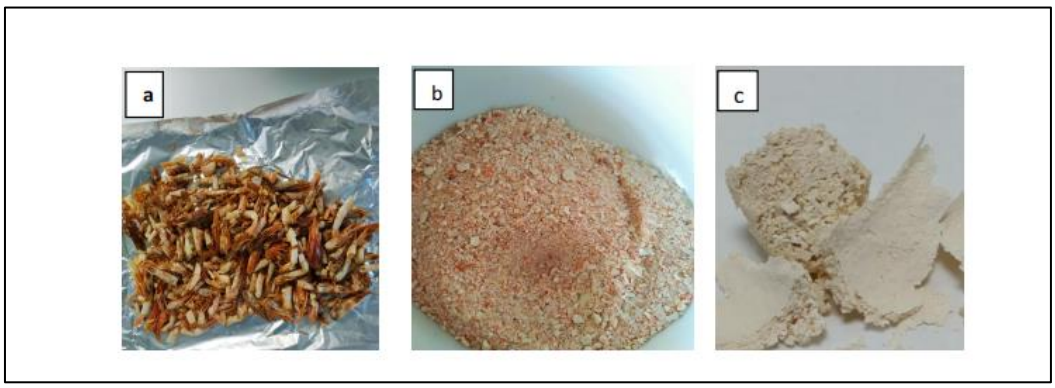

Figure 3 (a) Shrimp shells (b) after drying and grinding (c) chitosan

2.3. ITC Process

The interactions of three metals (Ca2+, Mg2+ and Zn2+) with chitosan were studied by ITC using the Nano ITC instrument (TA Instruments, USA). Although it is reported in the literature that chitosan can be dissolved in water, during the experiments it was found that the solubility of chitosan in aqueous solutions and in water is too low to perform ITC experiments [26]. In order to achieve complete dissolution of chitosan, a low pH is required. Chitosan stock solutions with concentrations from 0.5 mg/ml to 2 mg/ml in 1% acetic acid (pH 4.5) were prepared and used. In all runs, the metal was dissolved in the same solution as chitosan (1% acetic acid, pH 4.5). The chitosan solution was placed in the sample cell while the metal solution was placed in the syringe that performs the injections.

In a typical experiment, 25 injections of 5-10 μL aliquots of metal solution were added into the calorimeter cell with the chitosan. The titrations were continued with four to five additions after saturation was reached in order to determine the heat generated solely by ligand (background heat). A constant stirring speed (300 rpm) and temperature (25oC) maintained throughout to ensure proper mixing after each injection. Data analysis was performed using NanoAnalyze scientific plotting software according to model of the single set of identical independent sites. Each experiment was performed at least 3 times and the average Kd reported.

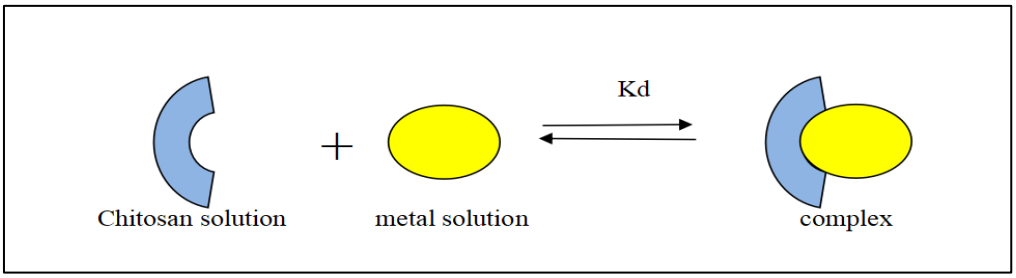

Figure 4 Flow diagram of ITC process of chitosan with metal

3. Results

Parameters and details of experiments are demonstrated in Table 1. Some differences can be observed attributed to the changes of molarity of solutions (NaOH, KOH, HCl) for deproteination, demineralization and deacetylation and to the ratio of solid to solvent. As a result, the percentage of yield differs in all experiments. Functional and physicochemical properties of chitosan that have been studied in this work have shown a variety of characteristics as it is demonstrated in Table 2. The color of chitosan is whitish slightly brown while the percentages of water binding capacity and oil binding capacity are 505 and 355, respectively. Chitosan shows high solubility in 1% acetic acid (93%) while values of ether extract and ash are below of 0.4%.

The percentage of yield of chitin and chitosan was presented in Table 3. Differences can be observed in the experiments. The percentage of yield of chitin varied from 14.9 to 19.2, the higher values were observed in exp II and IV while the lower value in exp III. Also, the percentage of yield of chitosan varied from 5.7 to 14.1, the higher value was observed in exp II while the lower value in exp III.

Chitosan is a linear polysaccharide and produced by the deacetylation of chitin, a process that removes acetyl groups from the N-acetylglucosamine units, resulting in the formation of free amino (−NH2) groups. Chitosan interacts with metal ions through several mechanisms, depending on the pH, metal ion species, and solution conditions. When pH is acidic, as the pH we used in our measurements (pH=4.5), the amino groups deprotonate, making them available for chelation. Chelation is the most dominant mechanism for cationic metal ions. The lone pair of electrons on the nitrogen atom of the amino group (−NH2) and/or the oxygen atoms of the hydroxyl groups (−OH) can coordinate with metal ions to form stable chelate complexes. This typically occurs at acidic pH where the amino groups are sufficiently deprotonated to act as ligands. Figure 5 shows the titration and binding curves obtained after subtracting the heat of dilution for the interaction between each metal and chitosan. The stoichiometry of divalent metals binding to chitosan (metal/chitosan molecule) as determined from ITC experiments varied for each different metal (number n in Table 4).

Table 1 Experimental details

Parameters	**Experiment**	**I**	**II**	**III**	**IV**	**V**
Molarity of solution for deproteination		1.0 N	2.0 N	1.0 N	2.0 N	1.0 N
Solid to solvent ratio		1:15	1:15	1:18	1:5	1:20
Molarity of solution for demineralization		1.0 N	1.0 N	1.0 N	1.0 N	1.0 N
Solid to solvent ratio		1:10	1:10	1:10	1:10	1:10
Molarity of solution for deacetylation		12.5N NaOH	12.5N NaOH	12.5N NaOH	12.5N NaOH	50% KOH
Solid to solvent ratio		1:10	1:10	1:10	1:10	1:10
Stirring period for Dp, Dm, Da Rpm		24h 250	24h 250	24h 250	24h 250	24h 250
Centrifugation (wbc, obc) Time		3500 rpm 30 min	3500 rpm 30 min	3500 rpm 30 min	3500 rpm 30 min	3500 rpm 30 min

Table 2 Physicochemical and functional properties of chitosan (values are expressed as Mean± S.D (n=5)

Parameters	**pH**	**Ether extract**	**Ash**	**Solubility in 1 % acetic acid**	**Wbc**	**Obc**	**Color**
Value	8.1±0.1	0.36±0.3	0.18±0.09	93±0.27	505±65.45	355±45.82	Whitish brown

Table 3 Yield % of chitin and chitosan

Exp.	**Shrimp shells (g)**	**Chitin (g)**	**Chitosan (g)**	**Chitin yield (%)**	**Chitosan final yield (%)**	**Exp.**	**Shrimp shells (g)**
I	8.5	1.53	0.75	18	11.3	I	8.5
II	8.5	1.63	1.2	19,2	14.1	II	8.5
III	7.50	1.12	0.43	14.9	5.7	III	7.50
IV	10.00	1.82	0.91	18.2	9.1	IV	10.00
V	7.00	1.29	0.76	18.4	10.8	V	7.00

Table 4 Thermodynamic Binding Parameters for Chitosan-Metal cation Interaction

Metals	**Kd*10^{-4}**	**n**	**ΔH (kJ/mol)**	**ΔG (kJ/mol)**	**ΔS (J/mol*K)**	**High Kd** ↓ **Low Kd**
Ca^{2+}	4.927 (± 3.752)	1.154(±0.384)	-0.205(± 0.110)	-18.88	62.63	
Zn^{2+}	3.735 (± 2.373)	0.873(±0.209)	0.492 (± 0.215)	-19.57	67.27	
Mg^{2+}	1.901 (± 1.579)	1.574(±0.163)	-0.2 (± 0.034)	-21.24	70.57	

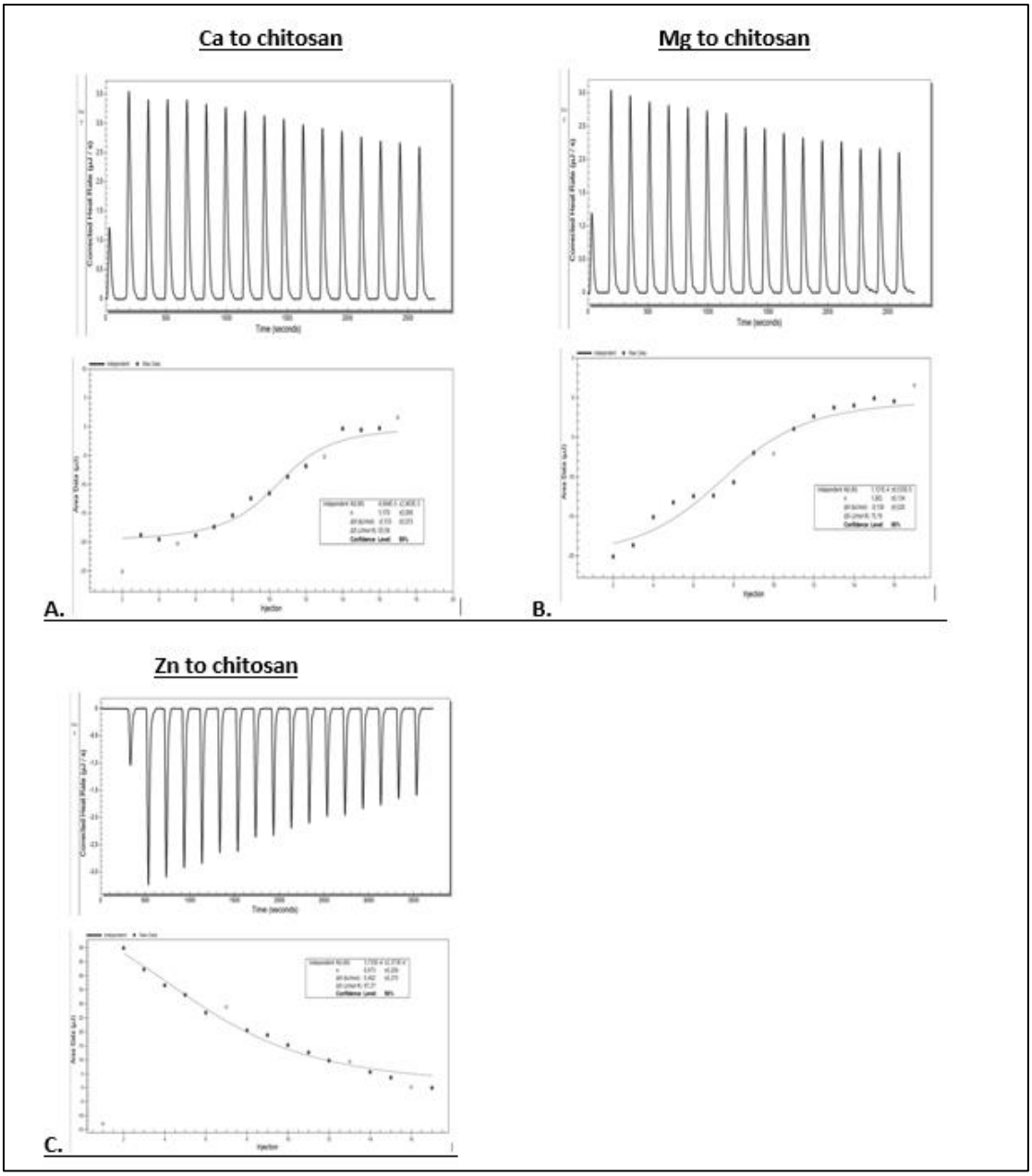

Figure 5 Isothermal titration calorimetry (ITC). Experimental data (upper panels) and binding isotherms (lower panels) obtained for: (A) Ca2+- chitosan, (B) Mg2+- chitosan, (C) Zn2+- chitosan

4. Discussion

Results from this work clearly demonstrate a variety of the percentage yield of chitin and chitosan. These values note the importance of the treatments of deproteination, demineralization (extraction of chitin) and deacetylation (extraction of chitosan) and can be attributed to the differences of molarity of solutions (NaOH, KOH, HCl) and to the ratio of solid to solvent. Concerning the parameters of the experiments and the percentage yield it is believed that the extraction process can be improved to gain higher yields of chitin and chitosan. In our experiment results of 1.0 N solution of HCl for demineralization, 2 N for deproteination and 12.5 N NaOH solutions for deacetylation at a solid to solvent ratio of 1:15, clearly demonstrate a significant yield of chitin and chitosan. Recovery of chitin and chitosan in the present study is similar to the yield from shrimp shell waste reported in other studies [1,4,8,9,22].

Functional and physicochemical properties of chitosan indicate a good quality product with valuable properties. Solubility is an important property to determine the quality of chitosan; high solubility refers to a good quality chitosan. Chitosan is soluble in dilute organic acids, like acetic acid or formic acid and insoluble in water and in basic pH solutions.

Its solubility depends on distribution of N-acetyl and free amino groups. Chitosan is protonated because of the presence of amino group in the aqueous acid solution which leads to its solubility. Also, higher values of solubility are combined with increasing degree of deacetylation due to removal of acetyl group from chitin. In our experiments, chitosan showed high solubility in 1% acetic acid (93%) and its whitish slightly brown color is similar to color of chitosan of other studies. The higher values of water and oil binding capacity of chitosan compared with reported studies found to be within the range. Finally, the lower values of the ash and ether extract content prove the purity of the sample indicating the completion of demineralization and deproteinization and confirm the quality of chitosan [1,5,9,22]. The ITC results for the three metals studied showed that their binding to chitosan is spontaneous, favorable and exothermic for Ca and Mg and endothermic for Zn (see Figure 5).

ΔS, a measure of disorder or randomness in a system, is crucial for understanding the driving forces behind molecular interactions, represents the change in entropy during a biomolecular binding event. The binding event between chitosan and metals is entropically favoured. The positive ΔS value of chitosan with Ca and Mg and Zn suggests that the binding event is driven by an increase in entropy, meaning the system becomes more disordered upon binding. This can occur when hydrophobic interactions are dominant, as water molecules are released from the binding interface.

ITC directly measures the heat released or absorbed (enthalpy change) when molecules bind to each other. This heat change is a direct consequence of the breaking and forming of chemical bonds during the binding process. The binding event between chitosan and Ca such as with Mg is enthalpically favoured.

The magnitude of ΔH can be influenced by several factors, including the strength of the interaction, the number of bonds broken and formed, and the presence of any coupled reactions. Negative value of ΔH of chitosan with Ca and Mg indicates favorable interactions, such as the formation of hydrogen bonds, van der Waals interactions, or hydrophobic interactions. Positive value of ΔH with chitosan - Zn complex suggests desolvation of binding partners, conformational changes, or changes in the ionization state of the molecules. The heat released is larger for the interaction between Mg and chitosan than for the interaction between Ca and chitosan. Gibbs free energy change (ΔG), is a thermodynamic quantity that determines whether the binding interaction between chitosan and metal will occur spontaneously under constant temperature and pressure. ΔG is related to enthalpy (ΔH) and entropy (ΔS) through the equation: $\Delta G = \Delta H - T\Delta S$, where T is the temperature in Kelvin. Negative value of ΔG indicates a spontaneous binding interaction, meaning the reaction favors the formation of the complex. The maximum negative value was observed between chitosan and cation of magnesium. Analysis of the interaction between metals and chitosan shows that there are significant contributions to Gibbs free energy of both an entropic and an enthalphic nature. However, the interaction is entropically favoured for all three metals tested (Table 4).

The n-value, also known as stoichiometry, is important for understanding the nature of the interaction, represents the number of ligand binding sites per macromolecule or the molar ratio of interacting molecules. The ratio of stoichiometry of divalent metals Mg complex was little higher than expected (see Table 4). Finally, Kd represents the dissociation constant, which is a measure of the strength of binding between two molecules, specifically the inverse of the association constant (Ka). The Kd is directly related to the Gibbs free energy of binding (ΔG) through the equation: $\Delta G = -R * T * \ln(Kd)$, where R is the gas constant and T is the absolute temperature. The smaller the Kd, the more tightly bound the ligand is and therefore the higher the affinity between the ligand and the receptor. The smaller Kd value was observed between chitosan and magnesium cation indicating a stronger interaction [17,18,20]. The overall order of binding affinities toward chitosan was determined to be as follows: $Mg^{2+} > Zn^{2+} > Ca^{2+}$. It is likely that the chitosan–metal cation complex formation occurs primarily through the amine groups functioning as ligands. The difference in magnitude of binding of chitosan with different metal cations is likely the result of a combination of several factors, including the ionic radii and the ionic strength of the metal cations and the geometry of the metal complexes. As a result, ITC results clearly demonstrate that chitosan is a versatile biopolymer that can effectively bind the three metals studied (Mg^{2+}, Zn^{2+} and Ca^{2+}).

5. Conclusion

In addition to attractive physical and mechanical properties, chitosan is becoming increasingly important natural polymer because of its unique combination of properties like biodegradability, biocompatibility and bioactivity. In the present study, chitosan was obtained from discarded crustacean exoskeleton as a white slightly brown powder with sufficient functional physicochemical properties. Due to its biocompatibility and ability to bind metal ions chitosan could gained attention as a potential adsorbent for heavy metal removal minimizing the impact of these elements on the aquatic ecosystem or on the industrial wastewater. Knowledge of kinetic and thermodynamic binding data for chitosan and heavy metal interactions could help with the removal of metal cations from contaminated solutions.

Compliance with ethical standards

Disclosure of conflict of interest

The authors declare no conflict of interest.

Author Contributions

Conceptualization, K.K. and M.R.; methodology, K.K. and M.R; software, M.R.; formal analysis, G.K.; investigation, K.K., M.R. and G.K.; resources, K.K.; data curation, K.K. and M.R.; writing—original draft preparation, K.K.; writing—review and editing, K.K., M.R. and G.K.; visualization, G.K.; supervision, K.K.; All authors have read and agreed to the published version of the manuscript.

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