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Biorefining of crab shells for protein recovery using natural deep eutectic solvents: physicochemical and functional characterization of crab shell proteins

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Seafood consumption has steadily increased in recent years. Among crustaceans, crabs have made a significant contribution to the food waste, due to 60% of shell which is inedible. Although crustacean shell valorization has gained significant attention, conventional approaches do not fully exploit the high-value potential of crab shells. In this study, cooked and uncooked snow crab shells were used as a feedstock for protein extraction. The protein content of cooked *versus* uncooked (fresh) crab shells ranged from 18.87–17.26% (dry weight). The proteins were extracted by using conventional alkaline extraction and Natural Deep Eutectic Solvents (NADES) assisted extraction. The highest extracted yield was achieved with combined NADES of Choline chloride (ChCl) and Malic acid, at a 1 : 30 solid to liquid ratio at 50 °C. Combined NADES of ChCl with Malonic acid and Lactic acid also demonstrated good performance, while the combined NADES of ChCl and glycerol was least effective. Across both cooked and uncooked shells, the highest protein concentration was achieved with ChCl-malonic acid, solid : liquid 1 : 30, and at 50 °C. Functional properties characterization results demonstrated that ChCl-malonic acid exhibited the most balanced performance, with high solubility, superior FC/FS (up to 125%), strong ESI at acidic pH, and enhanced thermal stability. Cooked proteins were more soluble than uncooked proteins across all NADES. However, cooked proteins were less heat-stable than uncooked proteins. Identifying the functional potential of these protein isolates highlights their suitability for food applications, transforming crab shell waste into a valuable zero-waste resource with utilization potential across all its components.

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Sustainability spotlight

This study advances the field of sustainable food processing by demonstrating the use of Natural Deep Eutectic Solvents (NADES) as a sustainable and green alternative to conventional alkaline reagents for protein recovery from crab shells. The key sustainability achievement of this study was achieving protein isolates with greater than 90% purity, by using biodegradable, non-volatile, nontoxic solvents under mild conditions (≤50 °C), while eliminating harsh chemicals. By extending the application of NADES beyond the traditional crab shell valorization for only chitin extraction, this research validates the sequential recovery of multiple components from a single feedstock (crab shell), which aligns with zero-waste valorization and circular bioeconomy principles. Future work should include improving process greenness through solvent recycling and integration of low-energy intensification strategies supported by life-cycle analysis and techno-economic analysis.

1 Introduction

Protein is a versatile biopolymer known for its functionality in human and animal nutrition, biomaterial development and environmental sustainability. With the increasing popularity of protein's functionality and role in economic growth and public health such as the aging population[1,2] the necessity of expanding the scope of protein research and introducing alternative protein sources into the food market becomes inevitable. Also, with the environmental concerns of land animal protein,[3] and dissatisfaction of some legume plant protein[4,5] marine proteins are top in the chart for emerging protein sources. Marine by-products are an abundant source of alternative protein source, which is accessible and cheap.[6] The seafood industry is one of the fastest growing food type with global fisheries and aquaculture production of 223 × 10^6 in 2022, representing a 4.4% increase compared to 2020.[7]

Globally, the marine (seafood) processing industry generates 8 million tonnes of shell waste each year, consisting primarily of

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crustacean exoskeletons rich in chitin, proteins, minerals, and pigments (carotenoid).[8] The Canadian commercial sea fisheries produced 878 547 metric tonnes of seafood in 2016 at an estimated value of CAD $3.4 billion. The total landed value of seafood consists mainly of shellfish which make up 82% of the total worth according to Fisheries and Oceans Canada (2017). Snow crab (*Chionoecetes opilio*), an important shellfish functions as the main species in this fishery because it receives extensive harvesting throughout Atlantic Canada especially in Newfoundland and Labrador, New Brunswick, and Nova Scotia. The snow crab species makes Canada one of the leading global producers and exporters of the species which generates substantial export income for the shellfish market.[9]

Crab shell, a common seafood byproduct is produced during the processing of both cooked and uncooked crabs. Cooked crab shell is a common waste from the culinary sector, including restaurants and hotels. Uncooked crab shell is the byproduct of crab meat processing factories. These food waste (culinary crab shell) and industrial byproduct from crab meat processing usually end up in manufacturing animal feed, soil fertilizer, and landfill covering material.[8] The disposal method for shells during processing creates environmental problems from the decomposition of their high carbon content (chitosan, protein) and inorganic content ($CaCO_3$). These constituents can be recovered and repurposed from landfill to high-quality concentrates. The process of extracting high-quality proteins from crab shells creates two advantages which include environmental protection and sustainable food ingredient supply.

The process of extracting proteins from crab shell proves to be difficult to accomplish.[10,11] The crab shell 'exoskeleton' exists as a fibrous composite structure that contains proteins bound to chitin fibrils while being embedded in a calcium carbonate matrix. Therefore, to recover the protein, intermolecular bonds between protein and chitin must be broken to release the protein molecules. The utilization of alkali solubilization in combination with isoelectric precipitation works well for protein extraction but produces denatured proteins, proteins with lost functionality, and creates toxic waste products.[12] The development of green solvents for protein extraction is a more sustainable approach to replace the conventional extraction method of alkali extraction. Natural deep eutectic solvents (NADES) are green solvents that have gained significant interest because they demonstrate the ability to extract proteins from complex matrices while minimizing environmental harm.[13] The combination of Choline chloride with organic acids or sugars or polyols creates NADES with the capacity to facilitate hydrogen bonding, salting out, and hydrophobic interactions.[14]

The selection of natural deep eutectic solvents (NADES) in this study was guided by considerations of food-grade safety, physicochemical properties, and efficacy in biopolymer extraction. Choline chloride was chosen as the hydrogen bond acceptor due to its low toxicity, biodegradability, and frequent use in food and pharmaceutical applications, making it suitable for sustainable food processing.[15] Organic acids such as lactic acid and malic acid were selected as hydrogen bond donors because their mild acidity can enhance protein solubility by disrupting protein–matrix interactions, which is beneficial for extraction. Glycerol was included as a hydrogen bond donor based on its high polarity, protein-stabilizing properties, and demonstrated effectiveness in improving extraction yields of polar biomolecules.[16] Malonic acid was selected as a hydrogen bond donor to represent a relatively stronger organic acid system and to evaluate the effect of higher acidity on extraction performance. Previous studies have reported that choline chloride-malonic acid-based deep eutectic solvents exhibited superior extraction efficiency for structural biopolymers from crustacean shells.[17] Collectively, these NADES combinations have a range of acidity, polarity, and viscosity, enabling systematic evaluation of solvent–protein interactions and optimization of extraction efficiency.

The NADES possess non-toxicity, thermal stability, simple preparation methods, and biodegradable properties which qualify them for applications in the food industry.[18] The application of NADES for extracting proteins from marine resources including crab shells has not received sufficient investigation although these solvents have proven effective for plant-based protein and phytochemical extraction.[19,20] Therefore, this research investigates a significant knowledge deficiency by studying the efficiency of NADES in protein extraction from processed snow crab shells (cooked and uncooked).

The main objective of this research is to identify the best favorable conditions for extracting proteins from snow crab shells through conventional alkaline extraction and environmentally friendly NADES extraction techniques and to identify extraction systems that yield proteins with enhanced functionality and stability for potential food applications. This research advances extraction science while supporting sustainability initiatives through its findings. The research follows circular bioeconomy principles by converting crab shell waste into valuable functional proteins. The research develops environmentally friendly processing techniques which show promise to decrease toxic chemical usage and create more sustainable seafood processing operations.

Besides extracted protein isolates were further analyzed to assess their physicochemical and functional properties, including solubility, foaming capacity and stability, emulsification activity index and emulsification solubility index, color, molecular structure (FTIR), and thermal stability (DSC) to determine whether certain extraction systems may produce proteins with enhanced functional characteristics. The functional properties of proteins determine their ability to interact with water and air and lipids which affects the final product texture, stability and sensory characteristics.[21] The degree of protein denaturation, molecular interactions and secondary structure changes during extraction becomes evident through thermal and structural analysis, which directly affect product stability and functional performance. The functionality evaluation helps food technologists to understand protein behavior in food formulation and processing environments. The knowledge of protein properties from crab shell waste enables their successful integration into multiple food products, such as foamed items, emulsified dressings and baked goods and high-protein beverages as a functional ingredient or partial protein replacements.

The conversion of seafood by-product waste into a valuable ingredient through valorization processes becomes possible due to this knowledge, which supports seafood by-product/waste valorization and circular bioeconomy initiatives. The study provides a comprehensive assessment of the properties of extracted proteins to establish their value in sustainable food development, while creating a connection between waste transformation and industrial implementation.

2 Materials and methods

2.1 Materials

Cooked Snow crab shell was collected from a local seafood restaurant in Montreal, Quebec. Uncooked crab shell was obtained from McGrew Seafood Inc. (Tracadie-Sheila, New Brunswick, Canada). Choline chloride (99.0%), glycerol (99 + %), malic acid (99 + %), malonic acid (99 + %) lactic acid (90 + %), citric acid (99 + %), NaOH (certified ACS pellets), ethanol, hydrochloric acid, petroleum ether were purchased from Fisher Scientific (Ontario, Canada). BCA Protein Assay Kit (Millipore Corp), Bovine Serum Albumin (BSA). All chemicals used were analytical grade.

2.2 Methods

2.2.1 Sample collection and preparation. Snow crab (*Chionoecetes opilio*) shells samples were collected from two distinct sources to reflect differences in processing: uncooked (fresh) shells, which had not been subjected to any heat treatment, and cooked crab shells, which had undergone heat treatment (thermal processing). All samples were packed in clean, airtight polyethylene bags and transported to the laboratory under chilled conditions (4 °C). Upon arrival, visible meat residues were manually removed. The shells were then washed thoroughly with istilled water to remove surface impurities.

2.2.2 Sample drying and pulverization. After cleaning the crab shells, the crab shell samples were freeze-dried using a benchtop freeze dryer (Model 75034, Labconco Corp., Kansas City, KS, USA) to remove residual moisture. The dried samples were then ground into a fine powder using a laboratory grain mill (KitchenAid Grain Mill Stand Mixer Attachment, Model: KGM, Web Code: 10409619). To facilitate efficient grinding and prevent thermal degradation, liquid nitrogen was used during the pulverization process. The resulting powdered samples were stored in airtight containers at −20 °C until further analysis.

2.2.3 Proximate composition analysis. Proximate compositional analysis was performed to estimate crude fat, crude protein, total ash content, and carbohydrate content of both cooked and uncooked freeze-dried crab shell samples on a dry weight basis according to the Association of Official Analytical Chemists[22](AOAC, 2000) method.

2.2.3.1 Determination of protein content. Crude protein content of the crab shell samples was determined using the Dumas combustion method, in accordance with AOAC Official Method 968.06.[22] Approximately 0.2–0.3 g of finely ground sample was weighed into a capsule and analyzed using a nitrogen/protein analyzer elemental analyzer (EA 1112, ThermoQuest Elemental Analyzer, CE Instruments, Italy). To improve accuracy beyond the standard factor (6.25), the nitrogen conversion factor was calculated using the amino acid profile and total nitrogen content of crab proteins, *via* the Kjeldahl Nitrogen Conversion Factor Calculator.[23]

$$\text{Protein percentage in crab} = \text{total nitrogen percentage} \times 4.29 \quad (1)$$

This specific conversion factor (4.29) was calculated based on the amino acid composition of snow crab (*Chionoecetes* spp.) proteins using the Kjeldahl nitrogen conversion factor calculator (Version 1.2.0), which estimates the nitrogen-to-protein factor from amino acid profile data. This species-specific approach improves the accuracy of protein estimation for snow crab shell-derived materials.

2.2.4 Pretreatments of cooked and uncooked crab shells before protein extraction

2.2.4.1 Demineralization. Demineralization was performed to remove inorganic materials, primarily calcium carbonate ($CaCO_3$), from the crab shells. 1 : 10, 1 : 15, and 1 : 20 solid-to-liquid ratios of cooked and uncooked crab shell powder dissolved in 25% (w/v) citric acid solution, stirred at room temperature (25 °C) for 24 hours. The citric acid concentration used for this procedure was selected based on the method described by Tissera *et al.*[24] Then the solid residue and supernatants of the samples were separated by centrifugation (5000×*g*) at room temperature. The residue was washed repeatedly with distilled water until a neutral pH was achieved. The demineralized samples were oven-dried at 30 °C until they reached a constant weight.[24] After demineralization, demineralized samples were placed in a muffle furnace at 550 °C to calculate the percentage of demineralization (%DM). Demineralization efficiency was performed according to the method by with slight modifications.[25]

$$\%\text{DM} = \frac{\text{mineral content before treatment} - \text{mineral content after treatment}}{\text{mineral content before treatment}} \times 100 \quad (2)$$

2.2.4.2 Defatting and decolorization. Following demineralization, the crab shell powders were subjected to defatting and decolorization using 95% ethanol. Crab shell powder was soaked in 95% ethanol (1 : 10 w/v ratio) and stirred continuously at room temperature by renewing the solvent in several intervals for 8 hours according to the method described by Ifuku *et al.*

with slight modifications.[26] After treatment, the samples were filtered, washed thoroughly with deionized water to remove residual ethanol, and dried at 30 °C until a constant weight was achieved. Completion of decolouration was assessed by visual observation. Color removal was visually monitored by observing the intensity of pigmentation in the mixture after each cycle. The initial solvent change displayed a deep reddish-orange color, indicative of high astaxanthin and carotenoid content. The pretreated powders were then stored in airtight containers at −20 °C until further use for protein extraction.

2.2.5 Conventional alkaline protein extraction from crab shells. Conventional alkaline extraction followed by isoelectric precipitation was carried out according to the methods described by Hewage *et al.* (2024)[27] with slight modifications. First, pretreated crab shell powder was dispersed in distilled water at a 1 : 10 (w/v) solid : liquid ratio, thoroughly mixed for 10–15 s, and homogenized at 14 000 rpm for 2 min. The dispersion was adjusted to pH 9.5 using 1 M NaOH, and protein was extracted at room temperature by using CORNING PC-6200 magnetic stirrer (25 °C, 180 rpm, 2 h) followed by centrifugation using SORVALL LEGEND X1R centrifuge (15 000×*g*, 20 min, 4 °C). Then, the supernatant was adjusted to pH 4.5 using 1 M HCl solution. The precipitated protein was centrifuged (15 000×*g*, 20 min, 4 °C) to collect the protein pellets, then washed twice using distilled water, freeze-dried, and stored at −20 °C.

2.2.6 Preparation of NADES. Four types of NADES were prepared using choline chloride (ChCl) as the hydrogen bond acceptor (HBA), and different hydrogen bond donors (HBDs) including malic acid (Mal), malonic acid (Malon), lactic acid (Lac), and glycerol (Gly). The specific HBA : HBD : H_2O molar ratios were selected based on previous studies done by Huang *et al.*,[28] Bradić *et al.*,[29] Fanali *et al.*[30] and Huang *et al.*[31] with slight modifications. Table 1 indicates the different molar ratios used in preparation of NADES.

The required amounts of all these HBA and HBDs were weighed based on their molar ratios. Each HBA and HBD pair along with distilled water were transferred into a glass beaker and mixed thoroughly. The mixtures were stirred continuously at 500 rpm on a water bath, the mixture was heated in a water bath facilitated by a magnetic hot plate stirrer at 80 °C for 3–4 hours until a clear, homogenous, and transparent liquid was formed, indicating complete eutectic formation. The resulting NADES were allowed to cool to room temperature, transferred into airtight glass bottles, and stored at room temperature until further use for protein extraction according to the method described by Hewage *et al.*[27] with slight modifications.

Table 1 Composition of NADES prepared for protein extraction

Type of NADES	Molar ratio (HBA : HBD : H_2O)
ChCl-malic acid	1 : 1 : 2
ChCl-malonic acid	1 : 1 : 1
ChCl-lactic acid	1 : 2 : 1
ChCl-glycerol	1 : 2 : 1

2.2.7 NADES characterization

2.2.7.1 Density of NADES. The density of NADES was determined by using the method described by Koh *et al.*,[32] gravimetrically using a 5 ml calibrated measuring cylinder. Each NADES sample was carefully filled to the calibration mark, ensuring the absence of air bubbles, and the mass of the filled cylinder was recorded using an analytical balance. The density (ρ) was calculated according to the following Equation.

$$\rho_{\mathrm{NADES}} = \frac{\text{mass of NADES}}{\text{volume of NADES}} \quad (3)$$

2.2.7.2 Fourier transform infrared spectroscopy (FT-IR) analysis. Fourier Transform Infrared (FT-IR) spectroscopy was performed to identify the functional groups and confirm hydrogen-bond interactions in the prepared NADES. Spectra were recorded using a Cary 630 FTIR spectrometer (Agilent Technologies, Santa Clara, CA,USA) with an ATR sampling module and Omnic software. Samples were scanned in the spectral range of 4000–400 cm^{-1}. For comparative analysis, spectra were recorded for each NADES as well as for the individual components. All spectra were baseline-corrected and normalized before interpretation.[32]

2.2.8 Protein extraction with NADES under different conditions. Protein extraction from pretreated crab shell powders was performed using the four prepared NADES solvents under controlled extraction conditions according to the method by Fernandes *et al.*[33] with slight modifications. The experimental variables tested included extraction temperature (40 °C and 50 °C), solid-to-liquid ratios (1 : 10, 1 : 20, 1 : 30 w/v), and a constant extraction time of 2 hours. Approximately 10 g of demineralized and decolorized crab shell powder was weighed into 500 ml conical flask. The appropriate volume of NADES was added according to the designated solid-to-liquid ratio. The mixture was first mixed thoroughly for 10–15 seconds, followed by homogenization using a high-speed homogenizer (Model 850, Fisher Scientific, Ottawa, ON, Canada) at 20 000 rpm for 2 minutes. Subsequently, the flasks were placed in a shaking water bath (SHEL LAB) set to either 40 °C or 50 °C. Extraction was performed at a constant shaking speed of 120 rpm while the extraction time was fixed at 2 h for all treatments. After extraction, the flasks were allowed to cool to room temperature (25 °C) and then centrifuged at 5000×*g* for 10 minutes at 4 °C. The supernatant was carefully decanted and stored for protein recovery.

2.2.9 Protein recovery from NADES extract. Following extraction, the protein-rich supernatant obtained from the NADES-treated crab shell samples was subjected to precipitation by disrupting the eutectic structure of the solvent system. According to the method described by Hadinoto *et al.*[34] deionized water was added to the supernatant at a 15 : 1 (v/v) water-to-extract ratio. The excess water disrupted the hydrogen bonding interactions between choline chloride and the respective hydrogen bond donor (malic acid, malonic acid, lactic acid, or glycerol), thereby destabilizing the deep eutectic solvent and precipitating protein fraction. The resulting slurry was then centrifuged at 5000×*g* for 10 minutes at room temperature, and

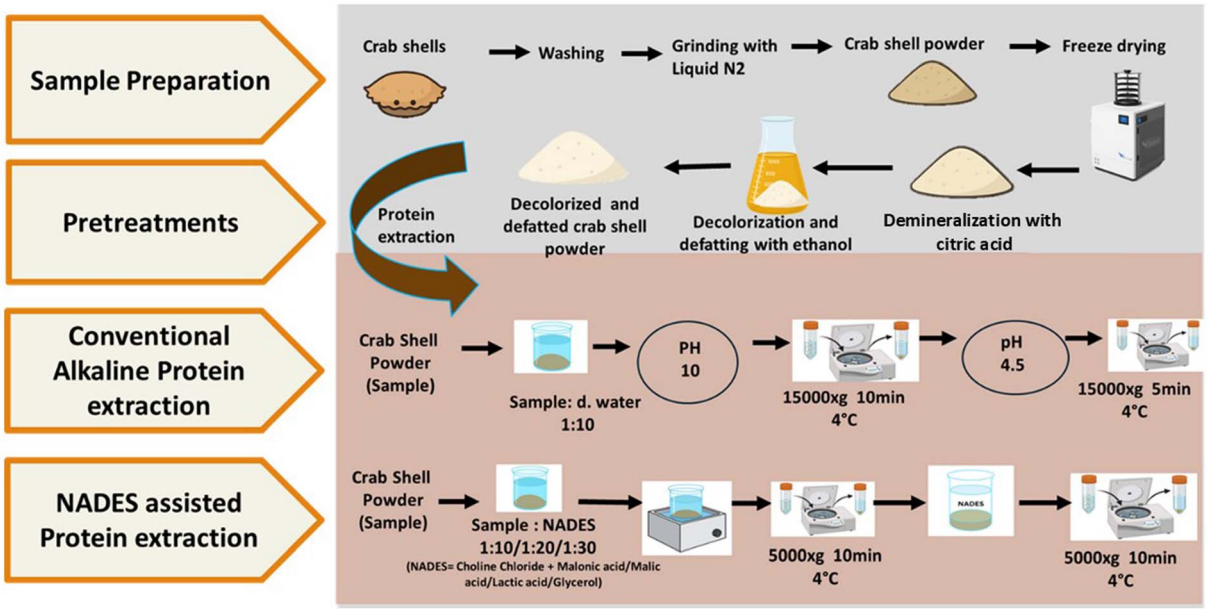

Fig. 1 Schematic illustration of the experimental workflow for NADES-assisted protein extraction from crab shells.

the sediment containing the precipitated protein extract was washed twice with deionized water to remove residual NADES components. The washed protein pellet was then freeze-dried. The final protein extract was collected and stored at −20 °C until further analysis.

Fig. 1 illustrates step by step methodology followed to extract proteins by using two different protein extraction methods.

2.2.10 Determination of protein yield. The dried protein powder is weighed using an analytical balance. The yield was calculated by using the following formula.

$$\text{Protein yield}(\%) = \frac{\text{mass of freeze dried protein(g)} \times 100}{\text{mass of crab shell powder used(g)}} \quad (4)$$

2.2.11 Determination of protein concentration in the protein extract. The protein content of crab shell isolates was determined using the bicinchoninic acid (BCA) assay. A standard curve was first prepared by serially diluting bovine serum albumin (BSA) to obtain concentrations of 31.25, 62.5, 125, 250, 500, 1000, and 2000 μg ml^{-1}. Freeze-dried crab shell protein extracts (1 mg) were dissolved in 1 ml of deionized water and vortexed thoroughly. For the assay, 25 μL of each standard or sample solution was mixed with 200 μL of BCA working reagent in a 96-well microplate, with all measurements conducted in triplicate. The BCA working reagent was prepared freshly as a 50 : 1 (v/v) mixture of Reagent A (BCA solution) and Reagent B (4% $CuSO_4 \cdot 5H_2O$). The plate was incubated at 37 °C for 30 min to allow for color development, after which absorbance was measured at 562 nm using a microplate reader.[35] Protein concentrations of the crab shell extracts were calculated by curve fitting from the BSA standard curve. The results were expressed as mg protein per ml of dissolved sample solution. The measured concentration (mg ml^{-1}) equals the protein mass (mg) dissolved in 1 mg of sample.

Protein content was expressed as % (w/w, dry basis) using

$$\% \text{ protein} = (\times \text{ mg ml}^{-1}/1 \text{ mg ml}^{-1}) \times 100 \quad (5)$$

2.2.12 Protein isolate characterization

2.2.12.1 Morphological characteristics of extracted proteins. The morphological characteristics of the crab shell powder, demineralized powders, and the protein obtained from different extractions were examined using scanning electron microscopy (SEM). The imaging was performed using the Hitachi TM-1000 tabletop microscope (Hitachi High-Tech Corporation, Tokyo, Japan) under high vacuum conditions using argon gas. Before imaging, a coating of 4 nm gold-palladium was applied by using a Leica EM ACE200 sputter coater (Leica Microsystems, Germany) under a vacuum with argon gas to make the surface conductive. An imaging resolution of 0.2 nm and an acceleration voltage of 1 kV were applied. For the measurements, 15 × kV accelerating potential and 500× magnification were used.[36]

2.2.12.2 Foaming properties. To determine the foaming properties, the following method described by Karimi *et al.*, 37was adopted with slight modifications. First 10 ml of cooked and uncooked crab protein suspensions (10 mg ml^{-1}) were made, and pH was adjusted to pH 3, 5, and 7 by using 0.1 M NaOH and 0.1 M HCl. Then those suspensions were homogenized at 15000 rpm for 2 minutes. The total volume of the sample was measured immediately after mixing and recorded. Again, after 30 minutes, another reading was taken at room temperature and recorded. Foaming capacity (FC) and foam stability (FS) were calculated using the following equations.

$$\text{FC}\% = \frac{(V_1 - V_0)}{V_0} \times 100 \quad (6)$$

$$\mathrm{FS}\% = \frac{(V_2 - V_0)}{V_0} \times 100 \quad (7)$$

V_0 = volume of the suspension before homogenization, V_1 = total volume immediately after homogenization, V_2 = total volume 30 minutes after homogenization.

2.2.12.3 Emulsifying properties. To determine the emulsifying properties, the following method described by Karimi *et al.*[37] was adopted with slight modifications. 6 ml of cooked and uncooked crab protein suspensions (10 mg ml^{-1}) were made, and pH was adjusted to pH 3, 5, and 7 by using 0.1 M NaOH and 0.1 M HCl. Then these suspensions were mixed with 2 ml of canola oil and homogenized at room temperature using a homogenizer at a speed of 25000 rpm for 2 min to produce emulsion droplets with a size of approximately 1–10 μm. 50 μL of fresh emulsion was taken from the bottom of the container and mixed with 5 ml of 0.1% SDS solution to measure the emulsification activity index. The turbidity of the mixture was measured using a UV-VIS spectrophotometer (Ultrospec, 2100 pro) at 500 nm. After 10 minutes of remaining at room temperature, another 50 μL of emulsion was taken from the bottom of the container, mixed with 5 ml of 0.1% SDS solution, and measured for turbidity at 500 nm to measure the emulsion stability index (ESI). Then EAI was calculated *via* the following equation,

$$\mathrm{EAI}\left(\mathrm{m}^2\ \mathrm{g}\right) = \frac{2 \times 2.303 \times A_0 \times \mathrm{DF}}{C \times \theta \times 10000} \quad (8)$$

A_0 = initial absorbance at 500 nm, DF = dilution factor (100), C = the protein concentration (g ml^{-1}), θ = volume of oil fraction (0.25).

ESI was measured by using the following equation,

$$\mathrm{ESI}(\mathrm{min}) = A_0 \frac{\Delta t}{\Delta A} \quad (9)$$

A_0 = the initial absorbance, Δt = the time between taking the first and second emulsion sample (10 min), ΔA = the difference in the absorbance of the samples.

2.2.12.4 Solubility. To determine the solubility properties, the following method described by Zhang *et al.*[38] was adopted with slight modifications. Cooked and uncooked crab protein suspensions with distilled water (10 mg ml^{-1}) were made, and pH was adjusted to pH 3, 5, and 7 by using 0.1 M NaOH and 0.1 M HCl. Then the suspensions were centrifuged for 15 minutes at 8000*g* at 4 °C. Then the protein concentration of whole solution and the supernatant were measured by using Biuret method.

$$\mathrm{Solubility} = P_s/P_w \times 100 \quad (10)$$

P_s = protein concentration of the supernatant, P_w = protein concentration of whole solution.

2.2.12.5 Color profile. The color profile of extracted protein isolates and flour was characterized using a color spectrophotometer (CM3500 d, Minolta Co. Ltd, Osaka, Japan). The sample was placed on the target mask in a Petri dish, and reflectance was measured at 2° standard observer and Illuminants based on L^*, a^*, and b^* values on the hunter scale.

2.2.12.6 Fourier transform infrared (FT-IR) spectroscopy. The molecular characteristics of cooked and uncooked crab shell proteins were analyzed using IR spectroscopy in triplicate. Infrared spectra were obtained at room temperature using a Cary 630 FT-IR spectrometer (Agilent Technologies, Santa Clara, CA, USA) with an ATR sampling module and Omnic software. Each sample was subjected to 64 scans with a resolution of 4 cm^{-1}, and the spectra were recorded from 400 to 4000 cm^{-1}. Proteins were analyzed for different amide bands and secondary structures to identify differences between different types of NADES-based extractions and different types of shells (Cooked and uncooked).

2.2.12.7 Differential scanning calorimetry (DSC). Thermal transitions of the protein fractions of proteins extracted from cooked and uncooked crab shells were evaluated by DSC (DSC 2500, TA Instruments, USA) according to the method described by Hewage.[39] Before testing, samples were dried over P_2O_5 in a hermetically sealed desiccator for 7 days. About 3–5 mg of the dried sample was weighed into hermetic aluminum pans and sealed. After equilibrating at 30 °C, scans were run from 30 to 250 °C at 10 °C min^{-1} under nitrogen (50 ml min^{-1}). Heat flow was recorded relative to an empty reference pan, and thermograms were processed using TA Universal Analysis 2000 software.

3 Statistical analysis

All measurements were conducted in triplicate ($n = 3$), and the results were expressed as mean ± standard deviation (SD). Statistical analysis was performed using one-way analysis of variance (ANOVA) to assess significant differences between sample groups. When a significant difference was observed ($p < 0.05$), Tukey's test was applied to determine pairwise differences among means. All statistical analyses were carried out using Minitab Statistical Software, Version 21 (Minitab LLC, State College, PA, USA).

4 Results and discussion

4.1 Proximate composition of cooked and uncooked shells

According to the proximate composition results of cooked and uncooked crab shells, there are significant differences observed in all components (Table 2). The moisture content of snow crab shells differed significantly between uncooked and cooked samples. Fresh shells contained 11.29% moisture (dry basis), whereas cooked shells contained 8.78% moisture (dry basis), resulting in a considerable decrease following thermal processing. This decline reflects heat-driven evaporation of free water, reduced water-holding capacity as proteins in the chitin–protein matrix denature and leaching of solubles into the cooking medium. During commercial processing of brown crab (*Cancer pagurus*), substantial cook loss was observed. Control samples (boiled in water) averaged 11.1% cook loss. This cook loss can occur due to evaporation of water during heating.[40] However, studies on crustacean processing show that the shell fraction tends to carry high water immediately after cooking, although absolute levels depend on species and sampling

Table 2 Proximate composition of cooked and uncooked crab shells[a]

	Crab shell composition (%) dry matter (dm)				
Source	Moisture	Proteins	Ash	Lipids	Carbohydrates
Cooked crab shells	8.78 ± 1.35^{g}	18.87 ± 0.12^{c}	55.12 ± 0.09^{b}	0.052 ± 0.009^{f}	17.178 ± 0.20^{d}
Uncooked crab shells	11.287 ± 0.18^{f}	17.26 ± 0.17^{d}	58.02 ± 0.24^{a}	0.15 ± 0.018^{f}	13.283 ± 0.11^{e}

[a] Values are mean ± SD (n = 3). Different superscript letters across the table denote significant differences between nutritional components of cooked and uncooked shells (p < 0.05; one-way ANOVA with Tukey's test).

conditions, which contradicts the data of this study. For instance, the moisture content of cooked Atlantic shrimp (*Pandalus borealis*) was approximately 74–76% moisture.[41] Table 2 presents the proximate compositional data of cooked and uncooked crab shells.

There could be several reasons other than thermal treatments for the different compositional data of crab shell such as anatomical compositional differences, and seasonal and molt-stage effects.[42] According to Moruf *et al.*,[43] crude fibre and carbohydrate (nitrogen-free extract) varied significantly between wet and dry seasons. At the same time, protein levels showed marked fluctuations during the wet season in the shells of the Portunid Crab (*Callinectes amnicola*). Compared with blue swimming crab shells, which have 18.14% protein, 29.78% ash, and 43.15% carbohydrate on a dm basis, the samples used in this research, which are snow crab, are more mineralized and show slightly lower protein and lower carbohydrates.[44] Besides mole crab contains 11.96% protein, 40.03% ash, and 13.82% carbohydrate (DW).[45] These values are lower than those in snow crab in this study in terms of carbohydrate, ash, and protein contents. These data indicate the compositional difference between crab species.

4.2 Demineralization efficiency

Demineralization was done to reduce the inorganic mineral load of the cooked and uncooked snow crab shells. In crab shells, the mineral portion is present in the form of calcite or amorphous calcium carbonate. It is deposited within the chitin–protein matrix, forming a unique structure. Ash content composed half of the dry weight of both cooked and uncooked crab shells. In previous studies of chitin extraction, demineralization has been done by using HCl.[46] However, due to the negative impacts of using harsh chemicals, later studies have been done by using mild chemicals such as citric acid, and malic acid.[24] Therefore, citric acid was used in solid-to-liquid ratios of 1 : 10, 1 : 15, and 1 : 20 to obtain the highest demineralization efficiency. From Fig. 2, the results show a clear influence of the solid-to-liquid ratio on demineralization efficiency for both cooked and uncooked crab shells. At the lowest ratio (1 : 10), efficiencies were moderate (90.32% for cooked; 90.42% for uncooked), but further dilution substantially improved mineral removal.

The highest efficiency was achieved at 1 : 20, where both cooked (97.91%) and uncooked (98.05%) shells reached the highest demineralization. Therefore, a 1 : 20 solid-to-liquid ratio was chosen for the demineralization before the process of protein extraction. By using a mild acid that minimally denatures or hydrolyzes protein, protein extraction was carried out using citric acid.

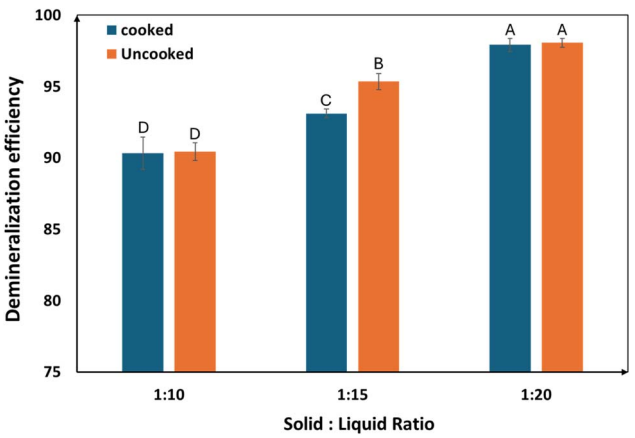

Fig. 2 Demineralization efficiency of cooked and uncooked crab shells using citric acid in different solid : liquid ratios.

4.3 Defatting and decolorization

Ethanol was used as the solvent for both defatting and decolorization because the fat content in both cooked and uncooked crab shells was relatively low. Thus, it was unnecessary to use stronger non-polar solvents such as petroleum ether or hexane, which are often applied in lipid removal. Ethanol, being food-grade, safe, and effective in dissolving residual lipids and carotenoid pigments such as astaxanthin, provided a more sustainable and milder alternative to harsh solvents.[47] Color removal was visually monitored by observing the intensity of pigmentation in the mixture after each cycle. Subsequent solvent replacements exhibited progressively lighter coloration, confirming the gradual reduction of residual pigments in the crab shell powders. Quantitative analysis of pigments before and after pigment removal was not done. However, the visible reduction in color intensity was considered as the qualitative evidence of effective pigment removal. According to visual observations, color pigment concentration was higher in uncooked crab shells than in cooked crab shells. It may be due to the loss of pigments while it was subjected to thermal treatment in cooked crab shells.

4.4 Characterization of NADES

The four NADES systems used in this study were designed by selecting choline chloride (ChCl) as the hydrogen bond acceptor (HBA) and combining it with different hydrogen bond donors (HBDs), including malic acid, malonic acid, lactic acid, and glycerol. These combinations were chosen based on their previously reported ability to form stable eutectic mixtures with favorable physicochemical properties for biomolecule solubilization and extraction according to the methods described by Huang *et al.*,[28] Bradić *et al.*,[29] Fanali *et al.*[30] and Huang *et al.*[31] with slight modifications. NADES made out of organic acids such as malic and malonic acid mixed with choline chloride provide strong hydrogen-bonding interactions and mild acidity, which have been shown to facilitate the disruption of the crab shell matrix made of proteins, minerals, and chitin, and thereby improving chitin recovery in previous studies.[31] Lactic acid was included as it not only contributes hydrogen-bonding capacity but also introduces mild acidity that enhances protein solubility under food-grade conditions. Abdallah *et al.* reported that lactic acid-based NADES had a higher capacity to isolate proteins, with extracts containing up to half of the total proteins present in mussel meat.[48] Glycerol, on the other hand, was selected as it offers high polarity and viscosity, which have been linked to protein stabilization and environmentally friendly extraction.[49]

In the present study, multiple reuse cycles were not experimentally evaluated, as the primary focus was on establishing the efficiency of the protein-first sequential biorefining approach. However, due to the non-volatile nature of NADES components, the solvent can be regenerated after aqueous extraction by removing excess water under reduced pressure (*e.g.*, rotary or vacuum evaporation) and restoring the original composition. Previous studies have demonstrated that such regeneration enables the recovery and reuse of NADES across multiple extraction cycles with minimal loss in extraction performance which support the feasibility of NADES recycling in the proposed system and highlight its potential to further improve the environmental and economic sustainability of the process.[50]

However, practical implementation of NADES recycling may be influenced by changes in solvent properties arising from residual water, co-extracted biomolecules, and mineral impurities, which can alter viscosity, pH, polarity, and hydrogen-bonding interactions, thereby affecting extraction efficiency and product purity in subsequent cycles.[51–53] Accordingly, further investigation of NADES stability and performance over repeated reuse cycles, including monitoring of key parameters such as viscosity, pH, extraction yield, and protein quality, is essential for validating large-scale applicability.

4.4.1 Density of NADES. Solvent density plays an important role in determining diffusion and miscibility with other liquids. Diffusion depends on the opportunity of molecules to move in the solvent. The ability of diffusion is comparatively lower in high-density solvents and higher in low-density solvents. In

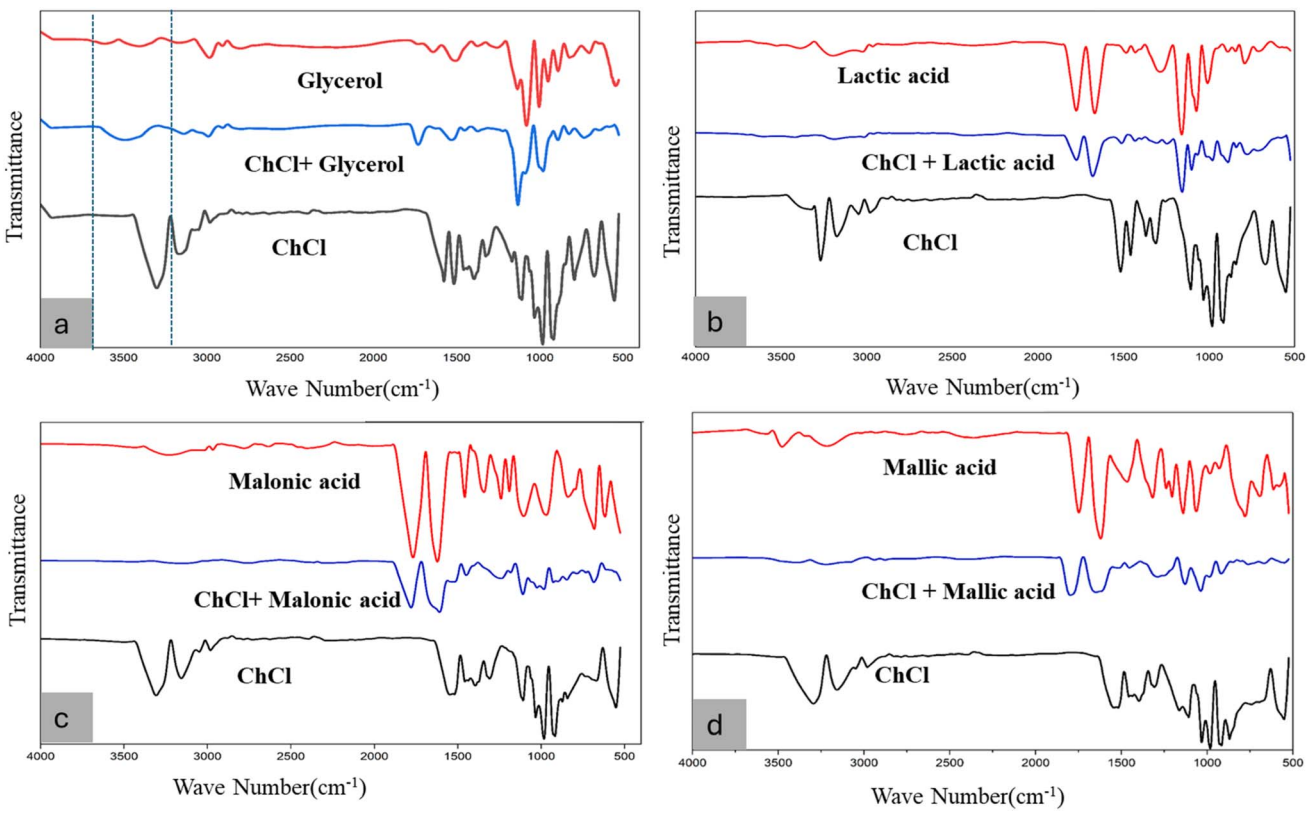

Fig. 3 (a) FTIR spectra of ChCl, glycerol, and NADES. (b) FTIR spectra of ChCl, lactic acid, and NADES. (c) FTIR spectra of ChCl, malonic acid, and NADES. (d) FTIR spectra of ChCl, Malic acid, and NADES.

NADES, density directly influences fluid mechanics and mass transfer, where higher densities of NADES may pose challenges for handling and mixing but can be beneficial for promoting phase separation.[54] The resulted densities of NADES were 1.189 g cm^{-3} for ChCl-malonic acid, 1.227 g cm^{-3} for ChCl-malic acid, 1.1402 g cm^{-3} for ChCl-lactic acid, and 1.150 g cm^{-3} for ChCl-glycerol. All four types of NADES showed a density above the density of water (1 g cm^{-3}). The results of the present study are well aligned with existing literature, further supporting the influence of hydrogen bonding on the relatively high densities of these systems compared to water. In a study by Biernacki *et al.* and Sazali *et al.* explained that, the density of ChCl : glycerol (1.20–1.22 g cm^{-3}) was slightly higher than that of ChCl : lactic (1.16–1.19 g cm^{-3}).[54,55] Both systems exhibited densities greater than water, which can be attributed to the extensive hydrogen bonding interactions within the DES.

4.4.2 FTIR of NADES. FTIR spectra were recorded for all five individual HBDs and HBAs, as well as for the four prepared NADES, at room temperature to characterize the types of interactions and the specific bonds involved. From FTIR graphs of these NADES with pure HBD and HBA, distinct peaks that are broadening and shifting were observed. Especially the O–H stretching region (3200–3600 cm^{-1}) was observed for H bonds formation. According to the existing data, changes in stretching vibration of NADES at the wavenumber of 3300 cm^{-1} are the main indication of hydrogen-bonding formation between HBA and HBD.[56] These results confirm the formulation of the NADES solvents with the increase and strengthening of hydrogen bonds. The observed shifts and broadened peaks provide clear evidence of hydrogen bond formation, confirming strong intermolecular interactions between the NADES components.[57] Fig. 3a present the FTIR spectra of glycerol, choline chloride, and the ChCl-Gly NADES. In the FTIR spectra, noticeable shifts in the broad O–H stretching region (3200–3600 cm^{-1}) confirm the presence of hydrogen bonding between glycerol hydroxyl groups and the chloride ion of choline chloride. These spectral changes indicate the formation of a strong hydrogen-bond network characteristic of NADES, consistent with previous studies.[58]

In the ChCl-lactic acid system, the broadening of the O–H stretching band (3200–3600 cm^{-1}) confirms strong hydrogen bonding between lactic acid functional groups (–OH) and the chloride ion of choline chloride, supporting the formation of a stable NADES network, consistent with previous reports.[30] The FTIR analysis confirmed strong hydrogen bonding between choline chloride and malonic acid, as evidenced by the broadening of O–H vibrations, indicating that carboxyl groups of malonic acid play a dominant role in stabilizing the NADES network[59]

The FTIR spectra of ChCl-malic Fig. 3d acid NADES revealed broadening and shifting of O–H bands confirming strong hydrogen bonding between malic acid functional groups and chloride ions of choline chloride, consistent with typical NADES formation mechanisms. According to previous studies, the FTIR spectrum of the ChCl-malic acid DES exhibits characteristic shifts confirming eutectic formation. Specifically, the O–H stretching band of choline chloride, initially reported at 3257.77 cm^{-1}, shifts to 3387.00 cm^{-1} in the DES, which is attributed to strong hydrogen bonding between the hydroxyl and carboxyl groups of malic acid and the chloride ion of choline chloride.[60]

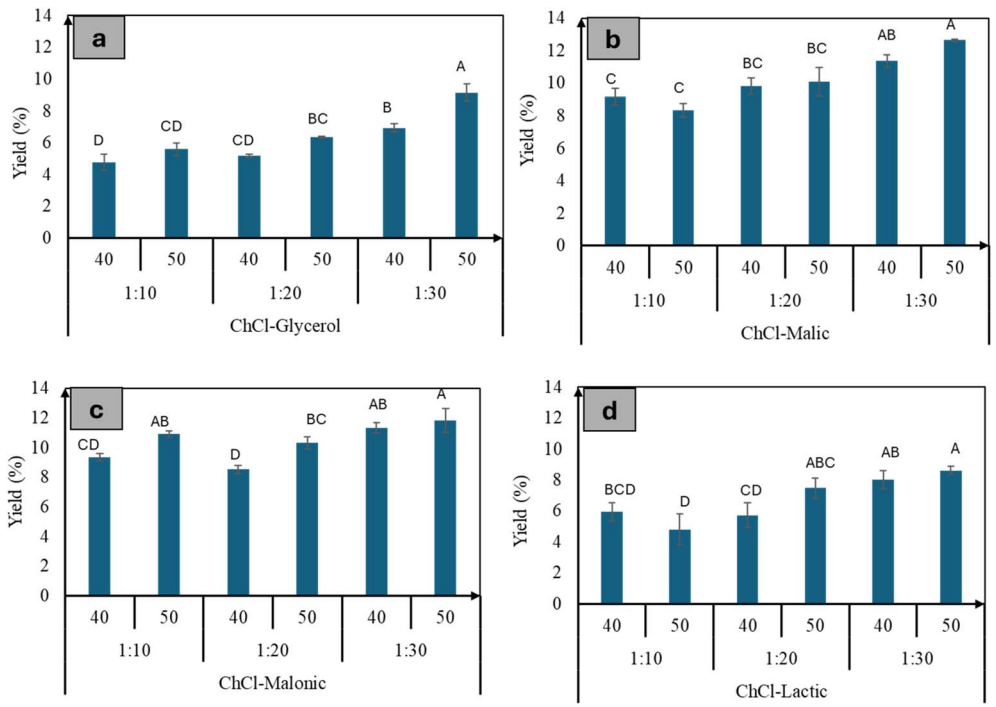

Fig. 4 Extraction yield (%) of obtained from uncooked crab shells using different solvents: (a) ChCl-glycerol NADES, (b) ChCl-Malic acid NADES (c) ChCl-malonic acid NADES, and (d) ChCl-lactic acid NADES. The extraction yield is expressed as % (w/w) relative to the dry crab shell powder.

4.5 Protein extraction and characterization

4.5.1 Extraction yield. Protein extraction was by conventional alkaline extraction as a control and natural deep eutectic solvents (NADES) under varied conditions. The protein extraction efficiency of different NADES systems from uncooked crab shells was evaluated under varying solid-to-liquid ratios and temperatures. Overall, both parameters had a significant influence on the extracted yield. Increasing the solid-to-liquid ratio from 1 : 10 to 1 : 30 consistently enhanced yields across all 4 NADES types, reflecting the importance of sufficient solvent volume for efficient solubilization. Similarly, higher temperatures (50 °C) generally improved extraction, likely due to reduced viscosity of the NADES and enhanced mass transfer, although minor fluctuations were observed in some treatments. Among the tested solvents, ChCl-malic acid exhibited the highest extraction efficiency, achieving a maximum yield of 12.64% at 1 : 30 and 50 °C, with very low variability (SD = 0.062). ChCl-malonic acid also demonstrated high performance, with yields above 11% under optimal conditions, though with slightly higher variability (SD = 0.808). In contrast, ChCl-lactic acid and ChCl-glycerol produced lower yields, with maximum recoveries of 8.62% and 9.15%, respectively, showing that these solvents are less effective for uncooked crab shells compared to their malic and malonic counterparts. Overall, the results indicated that the best combination of HBA and HBD for maximum extraction yield of proteins from uncooked crab shells is ChCl-malic acid at a 1 : 30 ratio and 50 °C. The extraction yield obtained from the conventional alkaline protein extraction method was 13.79% ± 0.85. Fig. 4 illustrates Extraction yield (%) of obtained from uncooked crab shells using different solvents.

Comparing this yield to 12.64% ± 0.06 obtained from the highest-performing NADES (ChCl-malic acid) at a 1 : 30 solid-to-liquid ratio and 50 °C, There was no significant difference in yield. This finding is important because it demonstrates that NADES can achieve comparable extraction efficiency to conventional methods while offering the additional benefits of being biodegradable, non-toxic, and environmentally sustainable. Other NADES, such as ChCl-malonic, also produced high yields (11.81%), though slightly below the control, while ChCl-glycerol and ChCl-lactic were less efficient. The results confirm that NADES, particularly ChCl-malic and malonic acids represent a promising green alternative for crab shell valorization, achieving similar efficiency to conventional solvents without compromising sustainability.

Fig. 5 illustrates extraction yield (%) of obtained from cooked crab shells using different solvents. The extraction of cooked crab shells using different NADES revealed that both the solid-to-liquid ratio and temperature significantly influenced yield. Increasing the solvent ratio from 1 : 10 to 1 : 30 consistently improved recovery across all NADES types. But raising the temperature from 40 °C to 50 °C generally enhanced efficiency due to reduced viscosity and improved diffusion. Therefore, the trend of the extraction yield of both shell types with increasing solid : liquid ratio and temperature is very similar. In cooked crab shells, ChCl-malonic and ChCl-malic provided the highest yields, with maximum recoveries of 13.18% and 12.87% at 1 : 30 and 50 °C, respectively. Statistical analysis confirmed that there was no significant difference between these two NADES,

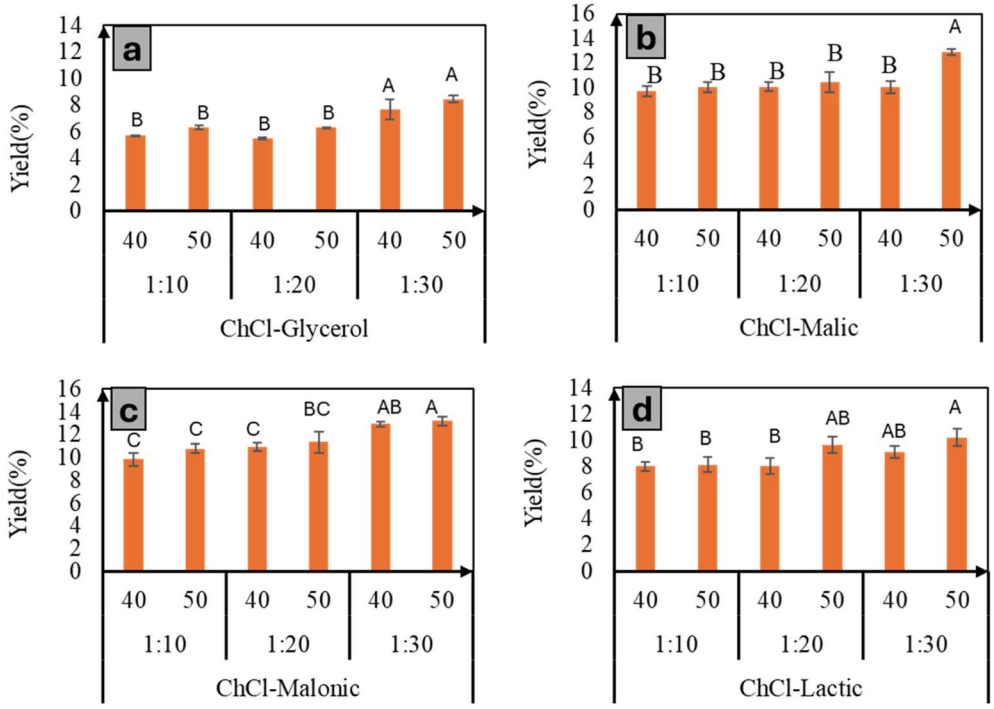

Fig. 5 Extraction yield (%) of obtained from cooked crab shells using different solvents: (a) ChCl-glycerol NADES, (b) ChCl-malic acid NADES (c) ChCl-malonic acid NADES, and (d) ChCl-lactic acid NADES. The extraction yield is expressed as % (w/w) relative to the dry crab shell powder.

Table 3 Protein concentration of cooked crab shell isolates[a]

NADES type	Solid : liquid ratio	Temperature	Protein concentration (%)
Alkaline (control)	1 : 10	25	66.28 ± 0.68
ChCl-glycerol	1 : 10	40	50.87 ± 0.42[b]
		50	51.14 ± 0.56[b]
	1 : 20	40	53.99 ± 0.36[a]
		50	53.59 ± 072[a]
	1 : 30	40	54.14 ± 0.26[a]
		50	54.46 ± 0.44[a]
ChCl-malic	1 : 10	40	73.61 ± 0.42[b]
		50	74.26 ± 0.36[ab]
	1 : 20	40	74.90 ± 0.51[ab]
		50	75.55 ± 0.48[a]
	1 : 30	40	74.90 ± 0.57[ab]
		50	75.41 ± 0.27[a]
ChCl-malonic	1 : 10	40	80.01 ± 0.40[d]
		50	85.72 ± 0.72[c]
	1 : 20	40	84.46 ± 0.52[c]
		50	86.01 ± 0.39[c]
	1 : 30	40	88.41 ± 0.58[b]
		50	91.00 ± 0.89[a]
ChCl-lactic	1 : 10	40	59.59 ± 0.79[e]
		50	66.28 ± 0.68[d]
	1 : 20	40	68.76 ± 0.71[c]
		50	70.63 ± 0.77[bc]
	1 : 30	40	71.74 ± 0.82[b]
		50	76.56 ± 0.44[a]

[a] Protein concentrations (as percentage %, mean ± SD) of proteins extracted from cooked crab shells using different NADES (ChCl-glycerol, ChCl-malic, ChCl-malonic, ChCl-lactic) and alkaline extraction under varying solid-to-liquid ratios (1 : 10, 1 : 20, 1 : 30) and temperatures (40 and 50 °C). Within each NADES type, values followed by different superscript letters are significantly different ($p < 0.05$, Tukey's test). Superscripts are not comparable across NADES types.

suggesting that both are equally effective for cooked crab shell valorization. Their strong performance can be attributed to the acidity and hydrogen-bonding capacity of malonic and malic acids, which likely enhance the disruption of chitin–protein structure in the shells. By comparison, ChCl-glycerol and ChCl-lactic exhibited lower efficiencies, with maximum yields of 8.43% and 10.21%, respectively. These differences may be due to their weaker acidity and less effective solvent–solute interactions, which reduce their ability to penetrate and solubilize the structural components of the shells. When compared to the conventional method (14.71% ± 0.68), both ChCl-malic and ChCl-malonic produced slightly lower yields, which were statistically significant. This indicates that while these NADES can approach the performance of traditional solvents, they do not yet fully match conventional extraction efficiency in terms of extraction yield in cooked crab shells. However, the advantages of NADES, such as biodegradability, non-toxicity, and environmental sustainability, make them promising green alternatives despite the reduction in yield.

According to these data, extraction yields from crab shells were influenced by both the state of the material, temperature, solid : liquid ratio, and the NADES composition. In cooked shells extracted protein yield is comparatively higher than the uncooked yield. It may be due to the disruption of protein–chitin structure, which increases solvent accessibility for enhanced protein extraction. Among the solvents, ChCl-malic and ChCl-malonic consistently gave the highest yields for both uncooked (12.64% and 11.81%) and cooked shells (12.87% and 13.18%). However, both were lower than the conventional methods (13.79% and 14.71% for uncooked and cooked, respectively). In contrast, ChCl-glycerol and ChCl-lactic produced lower recoveries, reflecting weaker solvent–matrix interactions. Overall, ChCl-malic and ChCl-malonic represent the most effective green alternatives, extracting highest protein yield from the crab shell powder compared to conventional solvents. The extraction temperature of 50 °C was selected based on its favorable effect on solvent viscosity and solute diffusion, which enhances mass transfer and extraction efficiency without causing excessive degradation. Previous NADES studies have also applied similar temperatures (around 50 °C) and reported improved recovery of proteins and bioactive compounds, as higher temperatures beyond this range were associated with decreased yields.[61]

4.5.2 Protein concentration of extracted isolate. The following Table 3 shows the different protein concentrations using the extraction methods under different conditions. Protein concentrations (as percentage %, mean ± SD) of proteins extracted from cooked crab shells using different NADES (ChCl-glycerol, ChCl-malic, ChCl-malonic, ChCl-lactic) and alkaline extraction under varying solid-to-liquid ratios (1 : 10, 1 : 20, 1 : 30) and temperatures (40 and 50 °C). Within each NADES type, values followed by different superscript letters are significantly different ($p < 0.05$, Tukey's test). Superscripts are not comparable across NADES types.

Table 4 Protein concentration in the uncooked crab shell isolate[a]

NADES type	Solid : liquid ratio	Temperature (°C)	Protein concentration (%)
Alkaline	1 : 10	25	68.15 ± 0.83
ChCl-glycerol	1 : 10	40	54.28 ± 0.97^{d}
		50	56.91 ± 2.33^{cd}
	1 : 20	40	56.47 ± 0.85^{d}
		50	60.86 ± 0.54^{bc}
	1 : 30	40	62.04 ± 0.79^{b}
		50	68.15 ± 0.84^{a}
ChCl-malic	1 : 10	40	76.63 ± 0.59^{b}
		50	77.20 ± 0.93^{b}
	1 : 20	40	78.36 ± 0.71^{b}
		50	79.00 ± 1.46^{b}
	1 : 30	40	84.49 ± 0.73^{a}
		50	85.43 ± 1.10^{a}
ChCl-malonic	1 : 10	40	84.61 ± 0.56^{c}
		50	85.69 ± 1.02^{c}
	1 : 20	40	87.34 ± 0.84^{bc}
		50	89.86 ± 0.93^{ab}
	1 : 30	40	90.82 ± 0.62^{a}
		50	92.26 ± 0.95^{a}
ChCl-lactic	1 : 10	40	60.93 ± 1.09^{c}
		50	61.93 ± 0.71^{c}
	1 : 20	40	71.20 ± 0.10^{b}
		50	72.28 ± 1.10^{ab}
	1 : 30	40	76.52 ± 2.76^{a}
		50	76.81 ± 0.81^{a}

[a] Protein concentrations (as percentage %, mean ± SD) of proteins extracted from uncooked crab shells using different NADES (ChCl-glycerol, ChCl-malic, ChCl-malonic, ChCl-lactic) and alkaline extraction under varying solid-to-liquid ratios (1 : 10, 1 : 20, 1 : 30) and temperatures (40 and 50 °C). Within each NADES type, values followed by different superscript letters are significantly different ($p < 0.05$, Tukey's test). Superscripts are not comparable across NADES types.

According to the data in Table 3, the protein concentrations obtained from cooked crab shells varied significantly depending on the NADES type, solid-to-liquid ratios, and temperature, with Tukey's test indicating clear differences within each solvent system. Among the four NADES examined, ChCl-malonic exhibited the highest protein concentration of the isolate, yielding values that increased steadily from 80.01 ± 0.40 at 1 : 10/40 °C to 91.00 ± 0.89 at 1 : 30/50 °C, demonstrating the strong influence of both temperature and solid : liquid ratio on protein recovery. ChCl-malic also produced consistently high protein concentrations (73.61 ± 0.42 to 75.55 ± 0.48), with significant differences between lower and higher ratios and temperatures. However, the overall protein concentration of the extracted isolate is lower in malic acid compared to malonic acid. ChCl-lactic showed intermediate performance, ranging from 59.59 ± 0.79 at 1 : 10/40 °C to 76.56 ± 0.44 at 1 : 30/50 °C, and ChCl-glycerol gave the lowest protein concentrations (50.87 ± 0.42 to 54.46 ± 0.44), significantly lower than the acid-based NADES. However, NADES which resulted low yield also improved extraction yield at higher solid : liquid ratios under higher temperatures.

The alkaline control produced a single value of 66.28 ± 0.68. It is comparatively higher than glycerol and the acid-based NADES. According to a study by Hong *et al.*, (2018) using NADES for extraction of chitin from lobster shell, deproteinization efficiency of acid-based DES depended strongly on the hydrogen-bond donor (HBD).[17] Across the systems tested, the order of effectiveness was ChCl-malonic acid (CCMnA) > ChCl-levulinic acid (CCLeA) > ChCl-malic acid (CCMA) > ChCl-lactic acid (CCLA). Consistently low ash levels in the resulting chitins indicate that these NADES pretreatments also removed calcium carbonate and other minerals effectively. Among the four, CCMnA produced the highest chitin purity, followed by CCMA and CCLA.[17] These results are well aligned with the data of the current study, as the high purity of chitin is due to the high efficiency of deproteination and demineralization, which indirectly causes high protein content. In addition to that, another study done by Zhu *et al.* (2017) reported that under comparable conditions, ChCl-glycerol (1 : 2, 100 °C) had no important effect on removing proteins or minerals, whereas ChCl-malonic acid (1 : 2, 100 °C) produced high-purity chitin and a higher yield (20.63 ± 3.30%) than the chemical control (16.53 ± 2.35%).[62] These results may be due to organic acid-based NADES outperforming neutral polyol systems due to their higher proton donation, stronger hydrogen-bond networks, and ability to disrupt associations in shell matrices.

Table 4 shows the protein concentration in the uncooked crab shell isolates. In uncooked crab shells, protein concentration increased with the higher solid : liquid ratios at 50 °C for all NADES. The numerical ranking at the optimum value is ChCl-malonic > ChCl-malic > ChCl-lactic > ChCl-glycerol, with highest yield of 92.26 ± 0.95 (malonic, 1 : 30/50 °C), 85.43 ± 1.10 (malic), 76.81 ± 0.81 (lactic), and 68.15 ± 0.84 (glycerol). Compared with cooked shells, the protein concentration of the

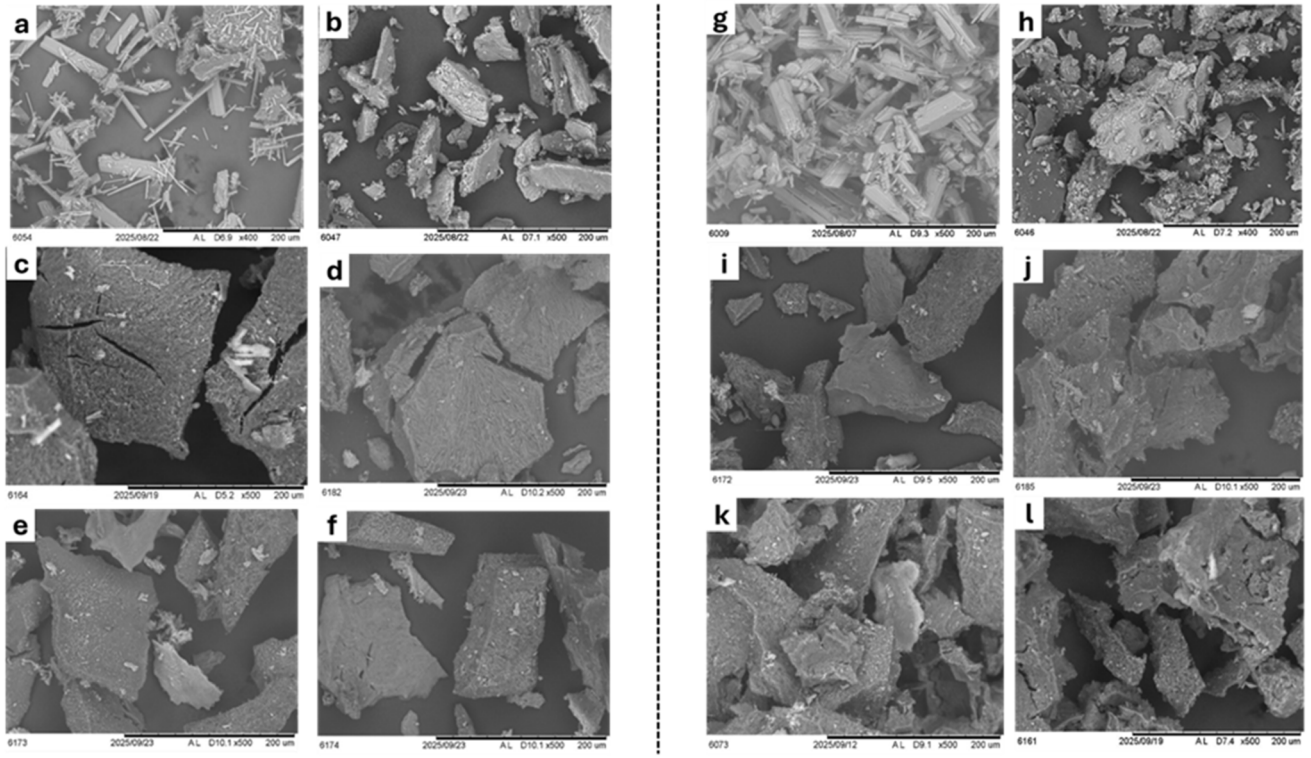

Fig. 6 Scanning electron micrographs of crab shell powders and protein extracts (all images at ×500; scale bar = 200 μm) (a) raw (uncooked) crab shell powder (b) demineralized uncooked shell powder (c) protein extract from uncooked shells-ChCl-glycerol NADES (d) protein extracts from uncooked shells-ChCl-malic NADES (e) protein extract from uncooked shells-ChCl-malonic NADES (f) protein extract from uncooked shells-ChCl-lactic NADES (g) cooked crab shell powder (h) cooked, demineralized shell powder (i) protein extract from cooked shells-ChCl-glycerol NADES (j) protein extract from cooked shells-ChCl-malic NADES (k) protein extract from cooked shells-ChCl-malonic NADES (l) protein extract from cooked shells-ChCl-lactic NADES.

extracted isolates using uncooked isolates was generally higher by using NADES and alkaline extraction. These results may be due to the denaturation of cooked shells. Uncooked shells likely retain more extractable/native protein and fewer heat-induced aggregates, while cooked shells can form intermolecular cross-linking and aggregation of protein molecules, generate larger particle aggregates and reduce the protein content in the isolate by decreasing the extractability of proteins from chitin matrix. Acid NADES (malonic > malic > lactic) showed the protein concentration, whereas neutral ChCl-glycerol is less effective. This trend was similar in both cooked and uncooked crab shells.

4.5.3 Scanning electron microscopy. Scanning microscopy images (Fig. 6a) shows the raw, uncooked shell has abundant rod-shaped crystals, assumed to be minerals such as $CaCO_3$ crystals.[63] Demineralized uncooked shell displays a more porous without needle shaped crystals, indicating effective mineral removal. For uncooked protein extracts, under different NADES showed differences in terms of their microstructure. None of the figures from 6c–f protein isolates show sharp needle-like or mineral crystals seen in raw shell powders. This confirms that demineralization successfully removed mineral fraction of crab shells. All these images show rough and cracked textures rather than the smooth surfaces, suggesting that proteins have undergone unfolding and partial denaturation. There is no evidence available in literature of SEM images from proteins extracted from crab shells. SEM micrographs of cooked crab shells (Fig. 6g) displayed distinct needle-like $CaCO_3$ crystals, similar to those observed in uncooked shells, but appearing more fragmented. This fragmentation may be because of cooking, which drives out water and soluble components. Previous studies have reported that thermal processing can enhance reorganization of structure by crystal fragmentation and increase pore exposure in crustacean shells.[64] Following demineralization, the sharp crystalline features disappeared, and the cooked shell samples exhibited irregular, porous, and rough-surfaced particles like in uncooked crab shells. However, compared to uncooked demineralized shells, the cooked samples appeared more collapsed and compact, likely due to heat-induced protein denaturation during boiling. For the cooked protein extracts (i–l), there are differences observed in protein structures extracted by using different NADES. Relative to uncooked extracts, cooked extracts appear more compact aggregates, consistent with heat-induced aggregation/compaction of proteins. Overall, SEM provides microstructural differences for the different extraction stages and protein isolates under different extraction mechanisms.

4.5.4 Foaming capacity and foam stability. Foaming capacity and foaming stability are key functional properties that serve as essential indicators to determine protein performance

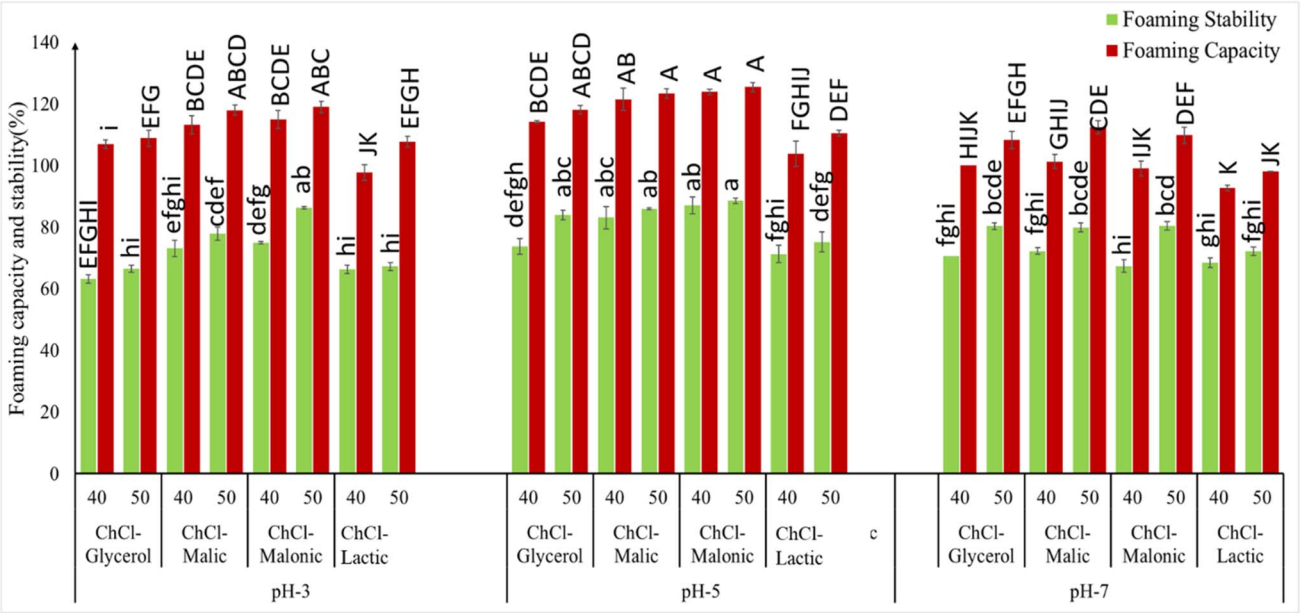

Fig. 7 Foaming stability (FS, green) and foaming capacity (FC, red) of proteins extracted from cooked crab shells using four NADES (ChCl-glycerol, ChCl-malic, ChC-malonic, ChCl-lactic) at 40 and 50 °C. Foams were prepared and evaluated at pH 3, 5, 7. Bars show mean ± SD; different letters denote significant differences by Tukey's test ($p < 0.05$) within each pH for the corresponding index (uppercase letters for FC, lowercase for FS).

in aerated food systems. In this study, both FS and FC generally increased with temperature from 40 °C to 50 °C across all NADES types as shown in Fig. 7 and 8. This trend is consistent with mild heating induces partial unfolding of proteins, exposing hydrophobic patches and flexible segments that migrate more rapidly to the air–water interface, increasing foaming capacity. This partial denaturation consists of alterations in the secondary, tertiary and quaternary structures.[65] Although previous studies have reported that a heat treatment applied, lower the foaming capacity of tarhana, which is a traditional Turkish cereal food compared to the untreated sample, this study showed improved FC and FS. The heat treatment above 55 °C appears to strengthen protein–protein interactions at the interface, likely through associations

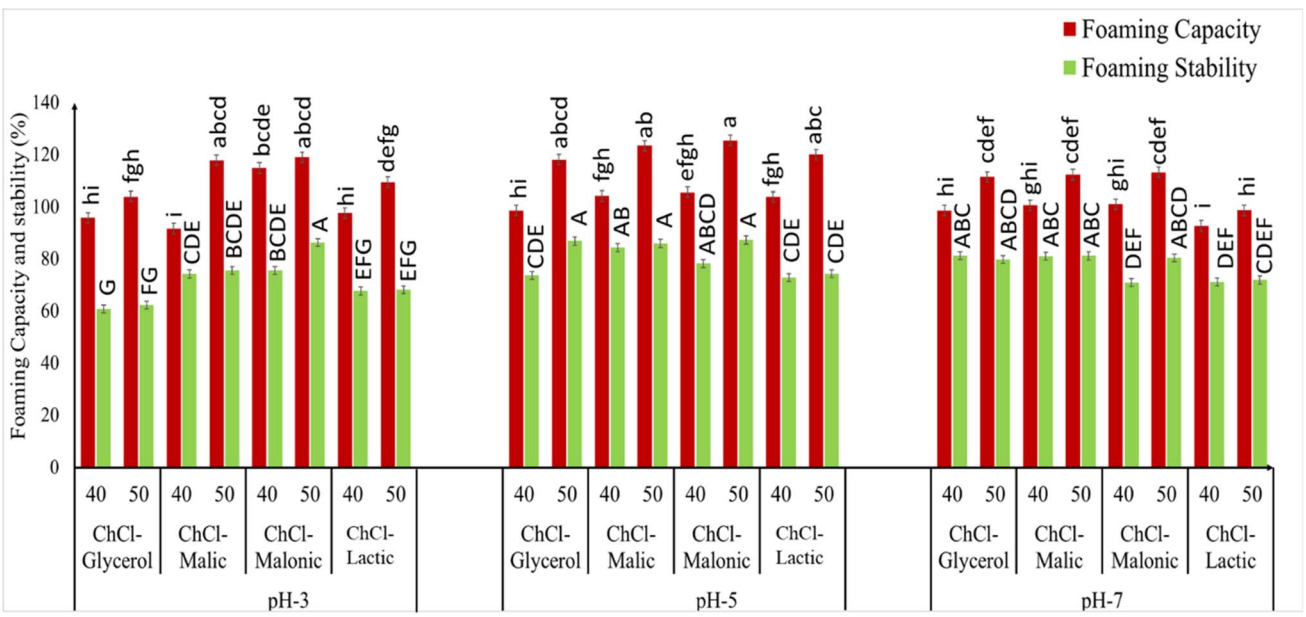

Fig. 8 Foaming stability (FS, green) and foaming capacity (FC, red) of proteins extracted from uncooked crab shells using four NADES (ChCl-glycerol, ChCl-malic, ChCl-malonic, ChCl-lactic) at 40 and 50 °C. Foams were prepared and evaluated at pH 3, 5, 7. Bars show mean ± SD; different letters denote significant differences by Tukey's test ($p < 0.05$) within each pH for the corresponding index (uppercase letters for FS, lowercase for FC).

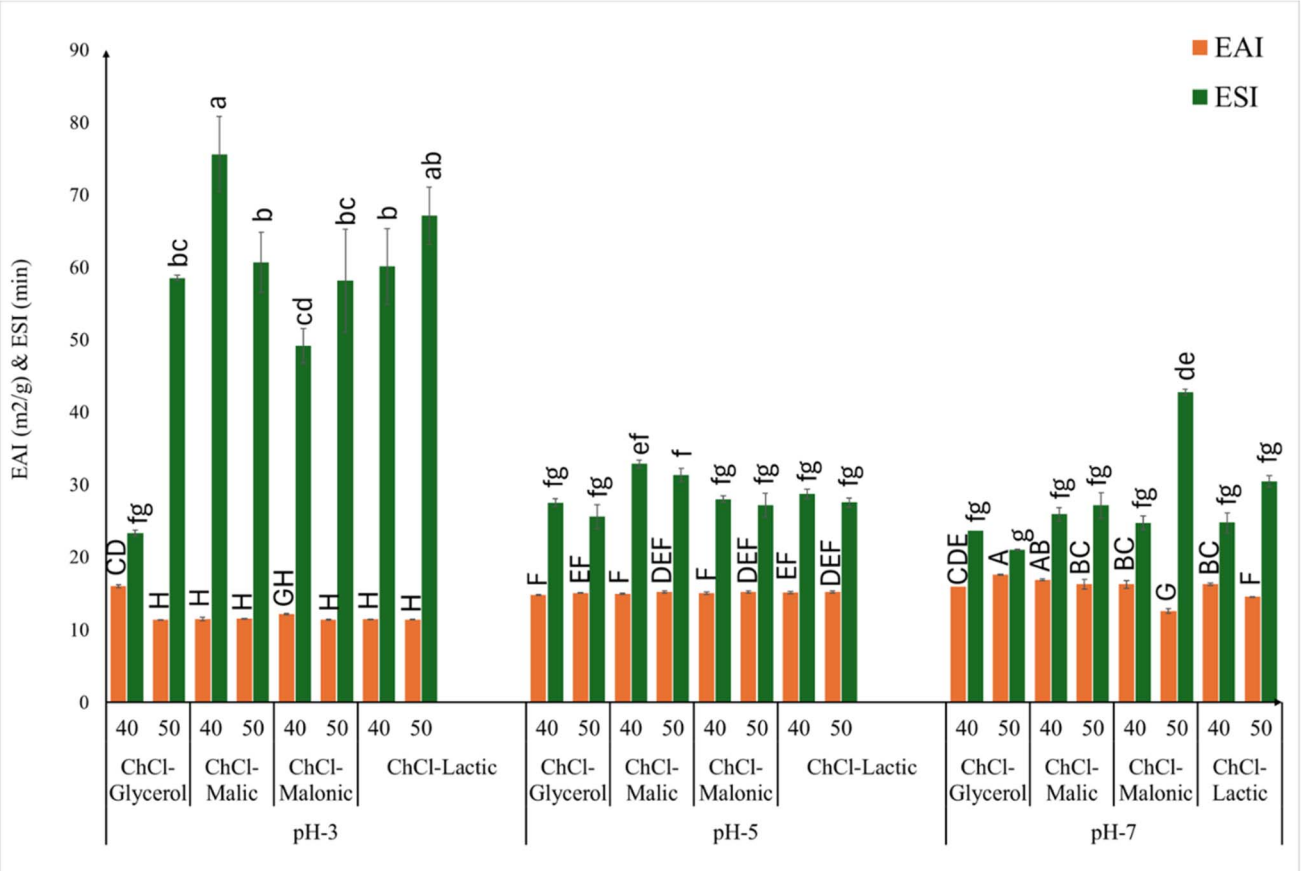

Fig. 9 Emulsifying activity index (EAI, orange) and emulsion stability index (ESI, green) of proteins extracted from uncooked crab shells using four NADES (ChCl-glycerol, ChCl-malic, ChCl-malonic, ChCl-lactic) at 40 and 50 °C. Emulsions were prepared and evaluated at pH 3, 5, 7. Bars show mean ± SD; different letters denote significant differences by Tukey's test ($p < 0.05$) within each pH for the corresponding index (uppercase letters for EAI, lowercase for ESI).

involving heat-exposed hydrophobic residues or sulfhydryl groups.[66]

Among NADES systems, ChCl-malonic consistently yielded the highest FS and FC reaching up to 88.60 (FS) and 125.48 (FC) at 50 °C under pH 5 in cooked crab shell proteins. This suggests that malonic acid-based NADES may enhance solubility and maintain structural flexibility of proteins, thereby maximizing interfacial activity. Previous studies have shown that acidic solvents can increase net positive charge, leading to stronger electrostatic repulsion between adsorbed proteins and more cohesive interfacial films.[67]

In terms of foaming capacity (FC), uncooked proteins generally exhibited superior performance, particularly at pH 5, where values reached as high as 125% in ChCl-malonic and ChCl-malic extracts at 50 °C. Foaming stability (FS) followed a similar trend, with uncooked proteins showing greater stability under pH 5, reaching 85–87%. The superior stability of uncooked proteins could be due to their higher solubility and optimal charge balance at pH 5, which promotes stronger protein–protein interactions and the formation of cohesive interfacial films. In both cooked and uncooked samples, pH 5 consistently supported the highest FC and FS, reflecting its position just above the isoelectric point of crab proteins which was 4.5. By contrast, at pH 3, excessive protonation restricted protein flexibility, leading to weak foam stability, while at pH 7, higher charge density reduced interfacial cohesion despite greater solubility.[68] Among the solvents, ChCl-malonic and ChCl-malic provided the best overall results, yielding high FC and FS in both cooked and uncooked proteins, likely due to their mild acidity and hydrogen bonding ability that maintained solubility and structural flexibility.

Considering the possible application of these foaming properties of extracted crab shell proteins, Foam-type cakes will be a better suggestion. In addition to that proteins with pH 3 for acid-stable foams with hydrocolloid support, and pH 7 for neutral whipped systems using the more stable extracts.[69] The conventionally extracted proteins followed the same temperature and pH trends but generally showed lower FC and FS values than the acidic NADES. For the conventional alkaline extract of cooked crab proteins, FC and FS were 92.05 ± 2.82% and 64.23 ± 1.49% at pH 3, increased to 100.33 ± 0.47% and 73.69 ± 2.52% at pH 5, and reached 92.05 ± 2.82% and 67.23 ± 2.82% at pH 7, with uncooked proteins exhibiting the same overall pH-dependent trend.

4.5.5 Emulsifying properties. The emulsifying activity index (EAI) and emulsion stability index (ESI) are key

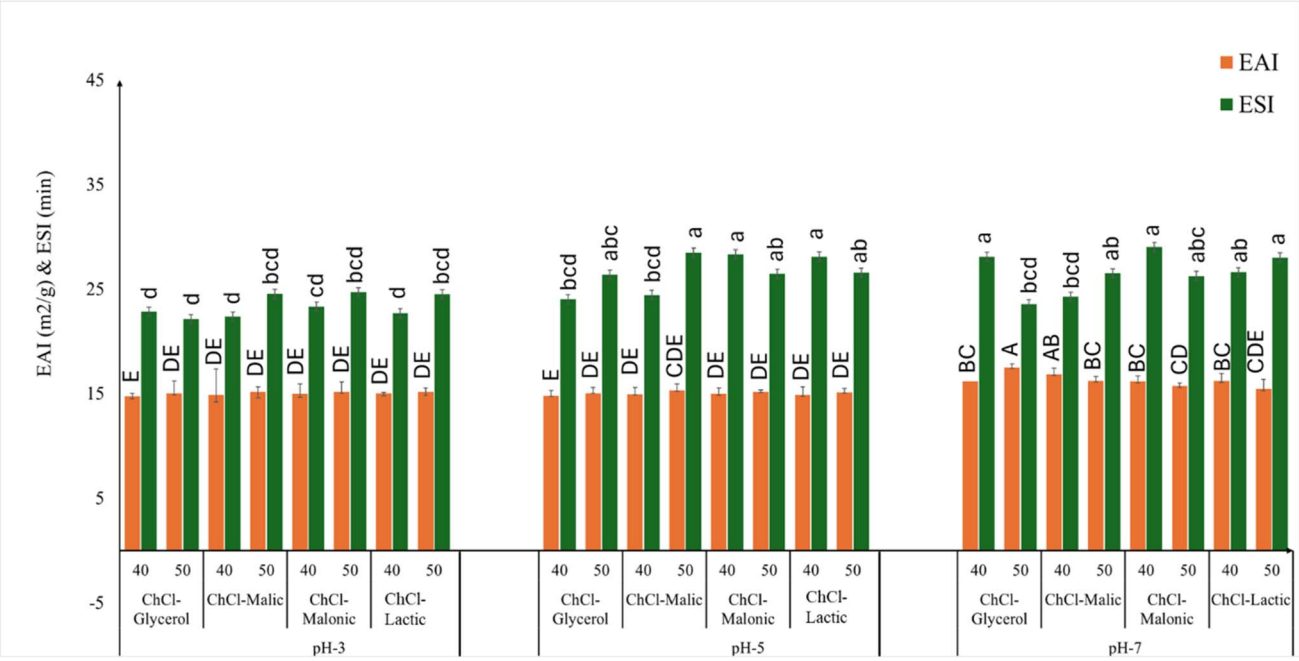

Fig. 10 Emulsifying activity index (EAI, orange) and emulsion stability index (ESI, green) of proteins extracted from cooked crab shells using four NADES (ChCl-glycerol, ChCl-malic, ChCl-malonic, ChCl-lactic) at 40 and 50 °C. Emulsions were prepared and evaluated at pH 3, 5, 7. Bars show mean ± SD; different letters denote significant differences by Tukey's test ($p < 0.05$) within each pH for the corresponding index (uppercase letters for EAI, lowercase for ESI).

parameters for evaluating protein performance in oil–water interface and maintaining emulsion stability by preventing droplet coalescence.[70,71] In this study, the emulsification activity index (EAI) and emulsion stability index (ESI) of cooked and uncooked proteins extracted using different NADES and conventional extraction were evaluated across pH 3, 5, and 7 (Fig. 9 and 10). The emulsifying behavior of the proteins recovered from uncooked crab shells depended strongly on pH, showing higher values at pH 7 than at pH 3 and 5. Most proteins extracted from all NADES had EAI in the range of 14.8–16.8 $m^2 g^{-1}$ at pH 5–7. The lower EAI at pH 3 and intermediate EAI at pH 5 indicate that EAI has a direct influence on the protein's isoelectric point. In this study, the isoelectric point of crab shell proteins was determined to be at 4.5 pH. Therefore, the pH values 3 and 5, which are very close to the isoelectric pH has low EAI compared to pH 7.

According to Kang *et al.*,[72] the green-crab protein extracted at pH 10 consistently delivered higher emulsifying activity (1482 $m^2 g^{-1}$) and markedly greater emulsifying stability than the protein extracted at pH 2 (858 $m^2 g^{-1}$), underscoring that extraction pH can precondition the proteins for superior interfacial performance at pH 7.5. It has been reported that larger peptides (>2 kDa) improve emulsifying properties because they unfold at the oil–water interface and contribute both hydrophobic and hydrophilic interactions, thereby enhancing emulsion stability through steric effects. However, smaller peptides diffuse faster to the interface and cover a wider surface area, but excessive hydrolysis can reduce amphiphilicity and weaken stability. Regarding pH, previous studies noted the lowest EAI and ESI near the isoelectric point (around pH 4 for soy and whey proteins) due to poor solubility, while maximal values were observed at alkaline pH (pH 8). This trend partly aligns with the results of this study, showing reduced emulsification close to the isoelectric pH. However, it also contradicts the data observed, that the most stable emulsions are at acidic pH (pH 3), not alkaline, indicating that in this study, stability may be driven by strong electrostatic repulsion and interfacial film rigidity under acidic conditions rather than at high pH.[73] However the ESI values showed a different trend, having higher ESI values at pH 3 and lower ESI at pH 5 and 7. This result contradicts the existing literature, which stated that the highest ESI values were observed in the lowest EAI, and ESI values were observed at pH 4, close to the isoelectric point of soy and whey proteins.[73]

In uncooked proteins, at pH 5, NADES types showed comparatively low ESI, ranging between 25–35 min, consistent with proximity to the isoelectric region where electrostatic repulsion between droplets is minimized and flocculation/coalescence accelerates. At pH 7, stabilities were generally lower ranging from 20–30 min, except for a ChCl-malonic at 50 °C, which achieved 42.8 min. Overall, proteins extracted from uncooked shells formed more stable emulsions than proteins from cooked shells in all pH conditions, especially at pH 3 with the acid NADES. For uncooked samples, ESI with ChCl-malic ranging from 75–61 min and ChCl-lactic ranging from 60–67 min, whereas cooked counterparts at pH 3 were much lower, around 22–40 min. The possible reason for this can be partial heat-induced unfolding exposes hydrophobic patches boosting

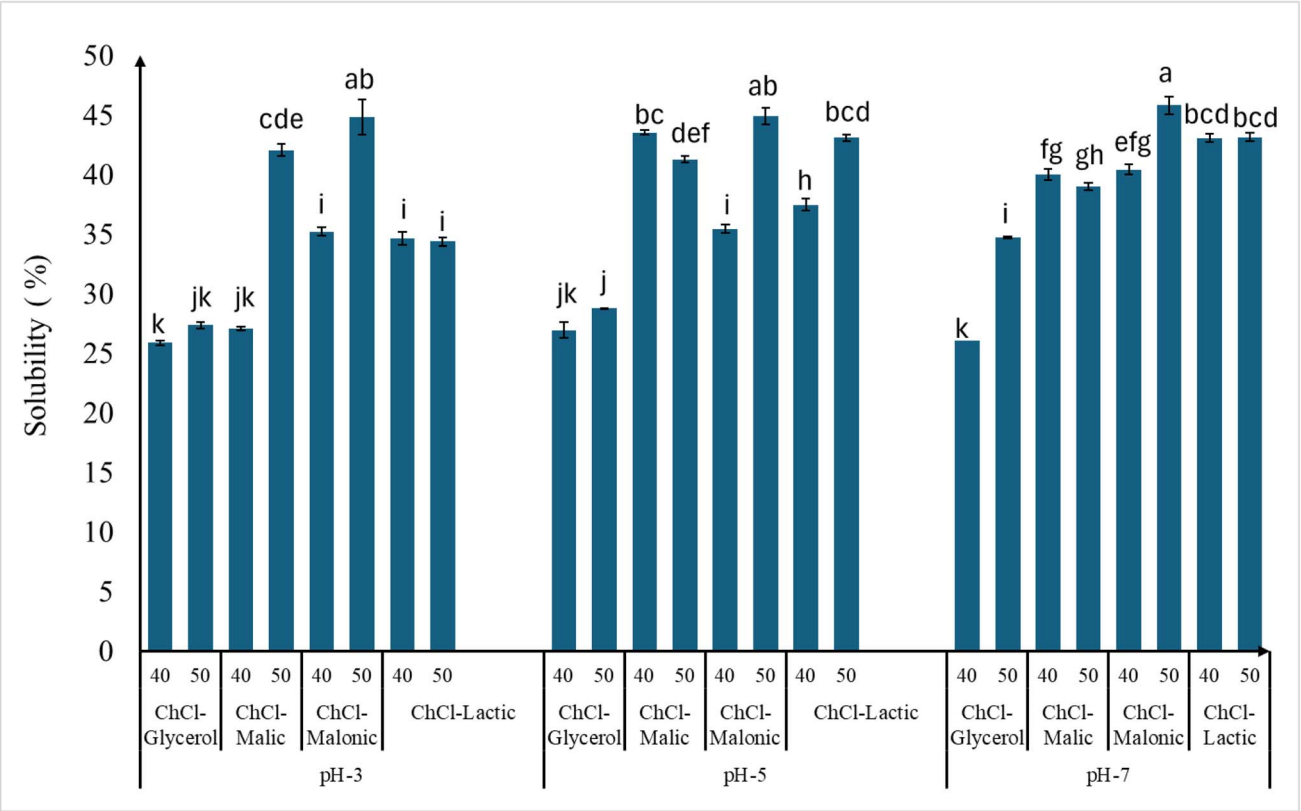

Fig. 11 Solubility of proteins extracted from uncooked crab shells using four NADES (ChCl-glycerol, ChCl-malic, ChCl-malonic, ChCl-lactic) at 40 and 50 °C. Solutions were prepared and evaluated at pH 3, 5 and 7.

interfacial adsorption (and thus EAI) rather than long-term film durability. In another study described that, at pH 7.5, the weaker flocculation yielded smaller, more mobile droplets that migrated upward more easily, resulting in lower stability. For the protein sample extracted at pH 12, stability was instead attributed to the formation of submicron-sized droplets combined with strong electrostatic repulsion at the oil–water interface, which prevented droplet aggregation and delayed creaming.[74]

Generally, it is recognized that heating can diminish proteins' emulsifying performance when denaturation and aggregation are extensive; by contrast, limited unfolding can enhance interfacial behavior and thus emulsifying ability. A study found that the EAI increased markedly after heat denaturation, while emulsion stability was essentially unchanged. However, the amount of protein adsorbed on fat droplet surfaces increased significantly, indicating that thermal unfolding and aggregate dispersion mainly boost interfacial loading (and thus EAI) rather than long-term film durability.[75] For comparison, the conventionally extracted uncooked proteins demonstrated EAI and ESI values that followed the same pH-dependent pattern observed in the NADES extracts. At pH 3, EAI was 11.32 ± 0.13 m^2 g^{-1} with an ESI of 51.07 ± 2.57 min, while at pH 5 the values increased to 13.16 ± 0.13 m^2 g^{-1} and 27.52 ± 0.63 min, respectively. At pH 7, EAI was 14.42 ± 0.02 m^2 g^{-1} and ESI was 17.04 ± 0.48 min. Cooked crab proteins extracted conventionally exhibited the same overall trend, although with generally lower EAI and ESI values due to heat-induced aggregation and reduced solubility. Overall, both cooked and uncooked proteins extracted with the conventional method have lower values than the NADES-extracted proteins.

In this study, the independent contribution of the NADES extraction medium to emulsifying activity (EAI) and emulsion stability (ESI) could not be resolved because pH and thermal history were the dominant determinants. The cooked and uncooked crab shell proteins both have high EAI at pH 7, but low ESI in uncooked crab shell proteins. This can be applied in the preparation of pre-emulsions for spray drying food systems, which do not have to be stabilized for a long period of time. The material used for spray drying will be responsible for the long-term stability and shelf life.[76,77] In addition to that, low EAI with highest ESI having uncooked crab shell proteins can be used for acidified food combined with polysaccharides such as pectin to reduce the effect of low EAI in acidic systems and to utilize high ESI in food systems.[78]

4.5.6 Solubility. For proteins in food systems, solubility is usually reported as the fraction of total protein that remains in the supernatant after dispersion and centrifugation at a defined pH/ionic strength.[79] In this study solubility of cooked and uncooked crab shell proteins were assessed under pH 3, 5 and 7 to check the potential of application into food systems. The solubility of crab shell proteins was significantly influenced by cooking, pH, NADES composition, and temperature (Fig. 11 and 12).

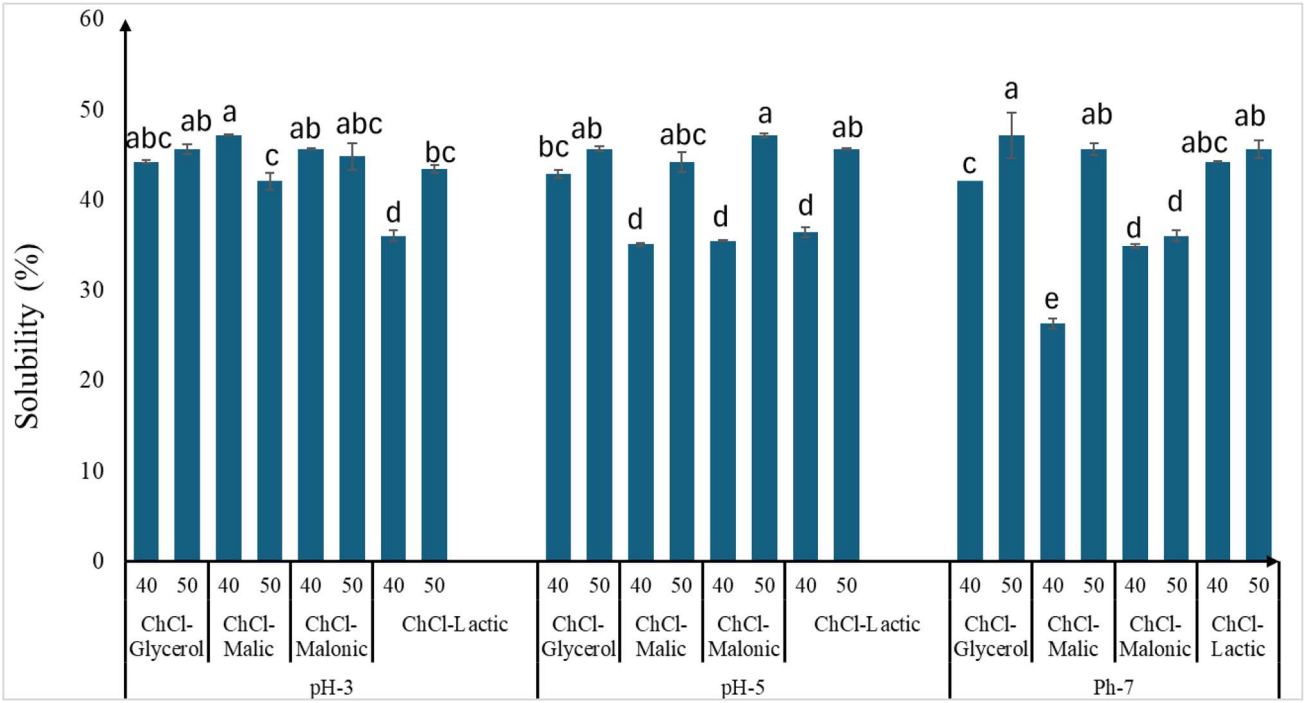

Fig. 12 Solubility of proteins extracted from cooked crab shells using four NADES (ChCl-glycerol, ChCl-malic, ChCl-malonic, ChCl-lactic) at 40 and 50 °C. Solutions were prepared and evaluated at pH 3, 5 and 7.

Cooked proteins exhibited higher solubility (35–47%) than uncooked proteins (25–45%), owing to partial unfolding during heating. The highest solubility was consistently observed at pH 5, just above the isoelectric point, where proteins carry a slight negative charge and remain more dispersible. Among the NADES, solubility of uncooked proteins extracted with ChCl-malonic, was improved with increasing temperature from 40 to 50 °C. Cooked proteins exhibited higher solubility (35–47%) than uncooked proteins (25–45%), owing to partial unfolding during heating. The highest solubility was consistently observed at pH 5, just above the isoelectric point, where proteins carry a slight negative charge and remain more dispersible. Among the NADES, solubility of uncooked proteins extracted with ChCl-malonic, was improved with increasing temperature from 40 to 50 °C.

The ChCl-malonic system had the highest solubility values across all pH (pH 3 : 44.84%; pH 5 : 44.91%; pH 7 : 45.85%). ChCl-glycerol was consistently lowest (≤34.71 at 50 °C), lactic and ChCl-malic were intermediate to high depending on pH. In cooked crab shell, proteins solubility is higher than uncooked crab shell proteins at all pH levels, and heating to 50 °C increased the protein solubility in all 4 NADES systems. However, the ChCl-glycerol extracted proteins showed lowest solubility for the uncooked crab shell proteins but increased protein solubility for the cooked shells, and at pH levels studied pH 3, 5, and 7. For the conventional alkaline extract of uncooked crab proteins, solubility at pH 3 was 25.71 ± 0.11% at 40 °C and 28.05 ± 1.81% at 50 °C. At pH 5, solubility values were 25.98 ± 1.15% (40 °C) and 26.99 ± 0.90% (50 °C), while at pH 7 they increased to 29.92 ± 3.73% and 32.53 ± 1.44% at the respective temperatures. These results show a slight temperature-dependent increase and the expected pH-dependent behavior, with solubility being highest at pH 7 and lowest near the isoelectric region. Cooked conventional extracts followed the same overall trend, displaying similar pH- and temperature-dependent solubility patterns.

Protein solubility is an important functional property for food systems because only soluble proteins can hydrate, diffuse, and adsorb to interfaces and thereby contribute to emulsification and foaming in food in all 4 types of NADES systems. Highly insoluble proteins show very poor emulsifying properties.[80] However, no strong correlation between solubility and emulsifying properties has ever been established. Solubility strongly influences the real application and function of an ingredient in a food system. If the solubility in different acidic and basic conditions is very poor, the applications of that ingredient in different food systems will be lower than ingredients with high solubility.[81] Solubility is governed by pH (relative to the isoelectric point), ionic strength, temperature, and protein structure. At pH 4 (close to many proteins' isoelectric point), the net charge is minimal, so electrostatic repulsion is weak and protein–protein interaction dominates. It causes the formation of large protein aggregates that can precipitate, lowering solubility. At pH values above and below the pI, proteins have more negative charge and experience stronger electrostatic repulsion, which keep molecules dispersed and therefore increases solubility.[82]

4.5.7 Fourier transform-infrared spectroscopy (FT-IR). To further understand structural integrity, Fourier-transform infrared (FTIR) spectroscopy was employed to identify

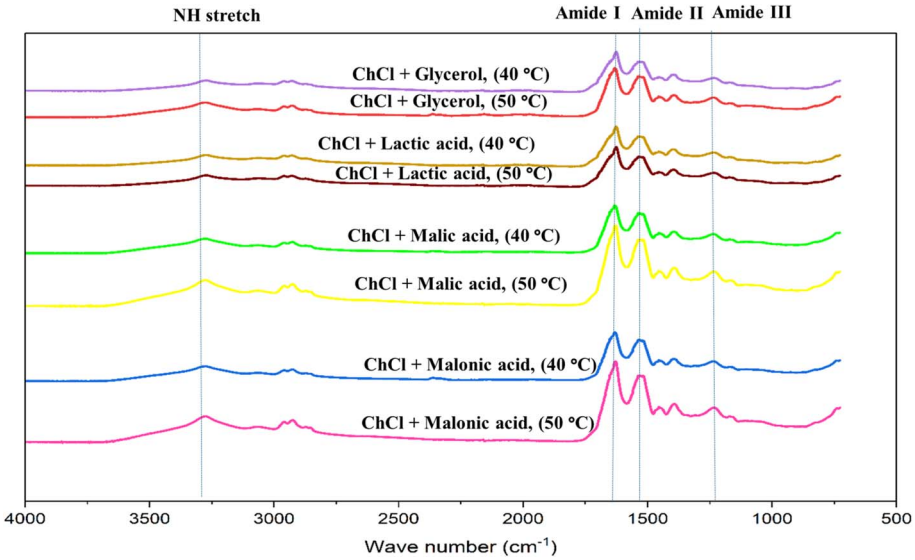

Fig. 13 FT-IR Spectra of cooked crab-shell proteins extracted with different types of NADES. choline chloride (ChCl) with Glycerol (Gly), Lactic acid (Lac), Malic acid (Mal), and Malonic acid (Malon). Each shown at 40 °C and 50 °C.

characteristic functional groups and secondary structure elements in the extracted proteins. Comparing the spectral features of cooked and uncooked extracts enables evaluation of heat-induced conformational changes, while variations among NADES types reveal differences in solvent–protein interactions and extraction selectivity. Proteins are often described by the proportions of their secondary-structure elements (α-helix, β-sheet, *etc.*). For proteins of unknown structure, this composition is among the most informative descriptors. Consequently, estimating secondary structure is a core application of FTIR spectroscopy.[83] According to the FTIR graph (Fig. 13) for cooked crab shell proteins extracted by using 4 types of NADES in two different temperatures, all the proteins display the dominant Amide I band near (1650 cm^{-1}) and Amide II near (1540 cm^{-1}), supported by a broad N–H stretch around 3300 cm^{-1} and weaker Amide III features (1300–1220 cm^{-1}).

This pattern confirms that the extracts are protein-rich and allows secondary-structure reading from Amide I shape. For instance, α-helix contributions centered ~1650–1658 cm^{-1}, β-sheet near ~1620–1630 cm^{-1} (Kong and Yu, 2007). The temperature increases from 40 °C to 50 °C produces minimal spectral changes. ChCl-glycerol and ChCl-lactic shows very little change in 40 °C and 50 °C. In the ChCl-malic and ChCl-malonic systems, Amide I becomes slightly broader at 1630 cm^{-1}, indicating a modest rise in β-sheet/aggregate structure. It can be assumed that under mild temperature conditions, no significant protein denaturation or major structural changes occurred, as indicated by the absence of strong band shifts or

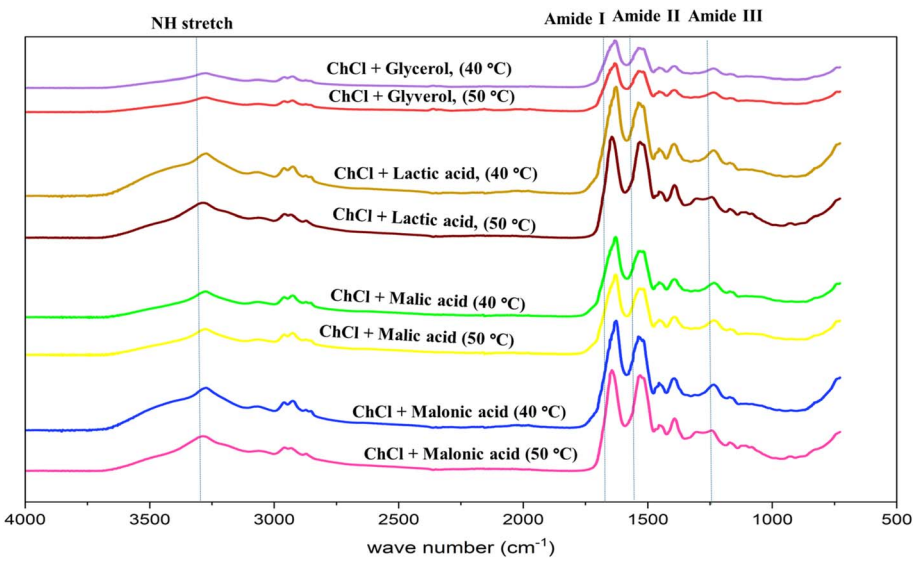

Fig. 14 FT-IR Spectra of uncooked crab-shell proteins extracted with different types of NADES. choline chloride (ChCl) with Glycerol (Gly), Lactic acid (Lac), Malic acid (Mal), and Malonic acid (Malon). Each shown at 40 °C and 50 °C.

Table 5 Color values of uncooked crab shell proteins

NADES type	Temperature (°C)	Color		
		L^*	a^*	b^*
Alkaline (control)	25	56.75 ± 0.411^{c}	-0.30 ± 0.06^{d}	14.17 ± 0.85^{ab}
ChCl-glycerol	40	54.33 ± 0.36^{d}	2.14 ± 0.12^{a}	15.58 ± 0.44^{a}
	50	54.32 ± 0.168^{d}	1.72 ± 0.10^{b}	8.31 ± 0.44^{d}
ChCl-malic	40	51.94 ± 0.196^{e}	1.05 ± 0.06^{c}	5.78 ± 0.09^{e}
	50	56.75 ± 0.411^{c}	-0.30 ± 0.06^{d}	14.17 ± 0.85^{ab}
ChCl-malonic	40	62.51 ± 0.645^{ab}	-0.25 ± 0.07^{d}	13.24 ± 0.96^{bc}
	50	60.88 ± 0.595^{b}	-0.23 ± 0.03^{d}	12.04 ± 0.31^{c}
ChCl-lactic	40	56.18 ± 0.853^{c}	-0.27 ± 0.01^{d}	15.53 ± 0.16^{a}
	50	63.38 ± 0.026^{a}	-0.28 ± 0.04^{d}	14.43 ± 0.31^{ab}

the appearance of new bands. IR-detectable reorganization of food proteins typically accelerates above 60–70 °C (*e.g.*, β-lactoglobulin/whey and milk-powder matrices), where β-sheet and β-turn content grow at the expense of α-helix. A separate study has been reported that FTIR showed noticeable changes in secondary structure of β-lactoglobulin, after subjected to above 70 °C and even at 90 °C some β-sheet remained, underscoring the higher threshold for extensive denaturation.[84]

All cooked extracts retained strong Amide I/II bands and increasing extraction temperature from 40 to 50 °C caused only minimal Amide I reshaping which are mostly evident in ChCl-malic acid and ChCl-malonic acid. FTIR supports limited conformational rearrangement at 50 °C rather than extensive thermal denaturation. All four solvent systems display the dominant Amide I band near 1650 cm^{-1} and Amide II near 1540 cm^{-1}, supported by a broad N–H stretch around 3300 cm^{-1} and weaker Amide III features at 1300–1220 cm^{-1}. This pattern confirms that the extracts are protein-rich. By comparing uncooked spectra (Fig. 14) with cooked spectra, cooked spectra show a broader, stronger band near ~3300 cm^{-1}, consistent with more hydrogen-bonding after heating. In the Amide I region (1600–1700 cm^{-1}), cooked spectra are broader and often display a low-frequency around ~1625–1630 cm^{-1} specially in ChCl-malic and ChCl-malonic, whereas uncooked spectra have a sharper peak near ~1652–1656 cm^{-1}. This may be due to partial unfolding/denaturation during cooking and thus rearrangement of secondary structures.

A major strength of IR spectroscopy for probing protein structure is that it doesn't depend on protein size or sample phase. Infrared spectroscopy is a staple for probing biological samples especially protein conformation. It is well established that peptide backbones give rise to nine characteristic amide bands: amide A (3300 cm^{-1}), amide B (3100 cm^{-1}), amide I (1650 cm^{-1}), amide II (1550 cm^{-1}), amide III (1300 cm^{-1}), amide IV (635 cm^{-1}), amide V (735 cm^{-1}), amide VI (600 cm^{-1}), and amide VII (200 cm^{-1}). Because these features originate from vibrations of the peptide bond (–CO–NH–), the IR spectrum carries detailed information about secondary-structure elements.[85,86]

By comparison, circular dichroism (CD) is also widely used to assess secondary structure, but it is largely limited to optically transparent, dilute solutions, which constrains its applicability relative to IR.[87] The amide I region (1600–1700 cm^{-1}), arising mainly from $C{=}O$ stretching of the peptide bond, is the most informative IR window for probing protein secondary structure. Because this vibration tracks backbone conformation and shifts differently for α-helices, β-sheets, turns, and coils FTIR can be used to identify and quantify secondary-structure content and monitor conformational changes in proteins and polypeptides.[88]

4.5.8 Color. Color analysis is a rapid, non-destructive method for protein quality and vital quality attribute that determines consumer acceptability of foods. Tables 5 and 6 show the color values (L^*, a^* and b^*) of uncooked and cooked crab shell – extracted proteins. The CIE Lab^* system objectively describes color in terms of lightness (L^*), red-green

Table 6 Color values of cooked crab shell proteins

NADES type	Temperature (°C)	Color		
		L^*	a^*	b^*
Alkaline (control)	25	61.62 ± 3.52^{b}	-0.72 ± 0.02^{e}	15.99 ± 0.64^{c}
ChCl-glycerol	40	58.79 ± 0.58^{b}	-0.21 ± 0.00^{d}	$14.09 \pm 0.03d^{e}$
	50	60.49 ± 0.2^{b}	-0.13 ± 0.05^{d}	13.16 ± 0.32^{e}
ChCl-malic	40	61.62 ± 3.52^{b}	-0.72 ± 0.02^{e}	15.99 ± 0.64^{c}
	50	63.38 ± 0.03^{a}	-0.28 ± 0.04^{d}	14.43 ± 0.31^{d}
ChCl-malonic	40	69.31 ± 0.06^{a}	0.86 ± 0.07^{a}	22.48 ± 0.39^{a}
	50	69.47 ± 0.11^{a}	0.65 ± 0.01^{b}	21.50 ± 0.34^{a}
ChCl-lactic	40	69.99 ± 0.09^{a}	0.45 ± 0.07^{c}	20.13 ± 0.10^{b}
	50	69.77 ± 0.03^{a}	0.41 ± 0.06^{c}	19.73 ± 0.06^{b}

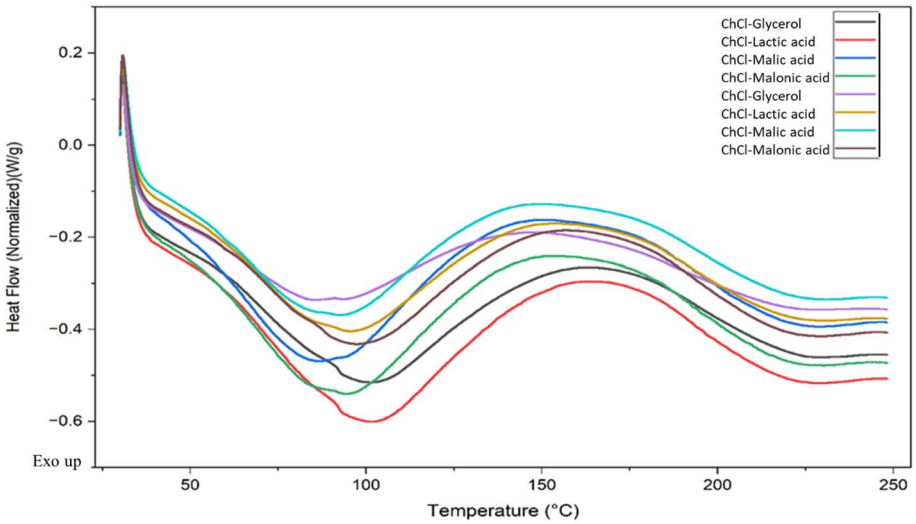

Fig. 15 Differential Scanning Calorimetry (DSC) thermograms (Exo up) of proteins extracted from cooked crab shells using four choline chloride (ChCl) based NADES (ChC-glycerol, ChCl-lactic, ChCl-malic, and ChCl-malonic at 40 °C and 50 °C. Samples A1–A8 correspond to: A1 = ChCl-glycerol (40 °C) A2 = ChCl-lactic (40°), A3 = ChCl-malic (40 °C), A4 = ChCl-malonic (40 °C, A5 = ChCl-glycerol (50 °C), A6 = ChCl-lactic (50 °C), A7 = ChCl-malic (50 °C) and A8 = ChCl-malonic (50 °C).

chromaticity (a^*), and yellow-blue chromaticity (b^*), and is widely used to assess pigment removal and processing-induced changes in food ingredients.[89] Across NADES types, cooked crab shell proteins extracted with ChCl-malonic and ChCl-lactic produced the lightest isolates (L^* ranged from 69–70), clearly higher than the alkaline control ($L^* = 61.62$).[90] The rise in L^* indicates more effective removal of impurities compared with the control. Organic-acid NADES can disrupt protein–chitin bondage and facilitate partitioning of carotenoids, yielding paler powders. Yang *et al.* (2025). Reported that, lightness after strong acid/alkali steps during crustacean shell processing exhibits better sensory effects.[90] a^* values moved from slightly greenish in the control ($a^* = -0.72$) to positive (reddish) for malonic/lactic systems (ranged from 0.4–0.9). This mechanism aligns with carotenoid chemistry in crustacean by-products and the known behavior of astaxanthin–protein complexes. In crustacean shells, astaxanthin, which is bound to proteins is called crustacyanin. Generally, carotenoproteins generate a wide range of colors from red and purple to blue/blue-black and yellow. While astaxanthin remains complexed with proteins and/or lipids (carotenoproteins or carotenolipoproteins), the tissue appears blue to green. However, when the complex is denatured or dissociates and free astaxanthin is released, the color shifts to reddish-orange.[91] Therefore, a^* value could be used as an indirect indicator of pigment-protein dissociation When the complex is disrupted by heat, acid, or solvent, astaxanthin is released in its free form, producing reddish-orange coloration and shifting the a^* value to positive. Therefore, a transition from negative to positive a^* values may reflect the degree of denaturation or dissociation of the carotenoprotein complex.

Yellow-blue (b^*) increased markedly with ChCl-malonic (ranged from 21–22) and ChCl-lactic (ranged from 19–20) *versus* the control ($b^* = 15.99$), indicating more yellowness. Temperature effects were minimal across the color profiles of cooked and uncooked crab shell proteins. In uncooked-shell proteins, solvent identity dominated color. ChCl-lactic (50 °C) and ChCl-malonic (40–50 °C) produced the lightest isolates (L^* ranged from 63.4 and 60.9–62.5), exceeding the alkaline control ($L^* = 56.75$), indicating more efficient removal of pigments in these organic-acid NADES. In contrast, ChCl-malic (40 °C) yielded the darkest/least light powders ($L^* = 51.9$) under 40 °C. Overall, organic acid-based NADES enhanced pigment removal and visual quality of crab shell protein isolates compared with alkaline and glycerol-based systems. Given that lighter-colored proteins are generally preferred for food formulation, particularly in neutral or lightly colored products, cooked crab shell proteins extracted with ChCl-malonic and ChCl-lactic show strong potential for food applications.[39] In that case, cooked crab shell protein isolates have higher potential for food application in terms of color.

4.5.9 Differential scanning calorimetry (DSC). Differential scanning calorimetry (DSC) tracks how a sample's heat capacity changes as temperature rises. For proteins, the resulting thermograms reveal thermal stability and act as a partial structural fingerprint that reflects conformational state. Using a DSC instrument, one determines the transition (melting/denaturation) temperature, and the enthalpy (ΔH) associated with disrupting the interactions that stabilize the protein's higher-order structure (Durowoju *et al.*, 2017). This DSC thermograms (Fig. 15) show the thermal stability of proteins extracted from cooked crab shell proteins extracted with four NADES. This also showed a single endotherm with a denaturation temperature ranging between 84.5–99.9 °C. At 40 °C extraction, the peak temperatures were between 86.61–99.85 °C, ChCl-lactic acid and ChCl-glycerol extracted proteins having the highest stability. At 50 °C extraction, the set shifted to 84.51–95.70 °C showing overall decrease of thermal stability,

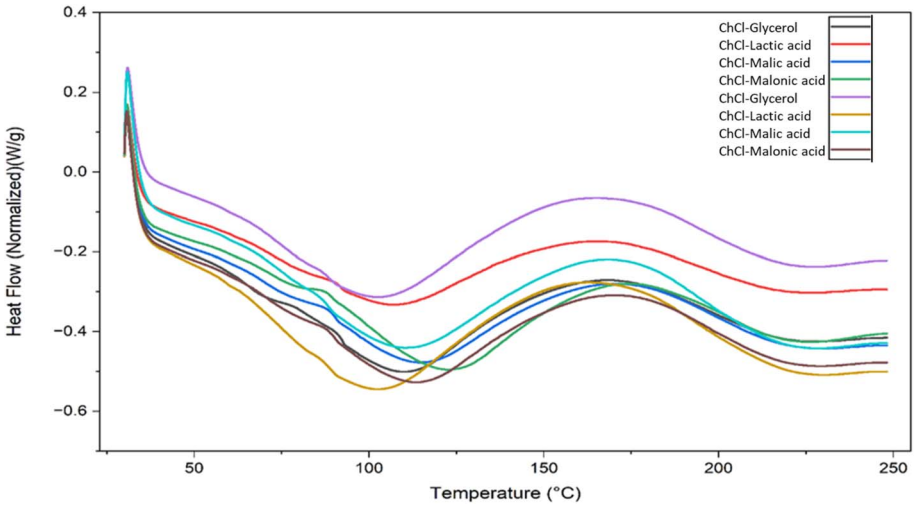

Fig. 16 Differential scanning calorimetry (DSC) thermograms (Exo up) of proteins extracted from uncooked crab shells using four choline chloride (ChCl) based NADES (ChCl-glycerol, ChCl-lactic, ChCl-malic, and ChCl-malonic at 40 °C and 50 °C. Samples A1–A8 correspond to: A1 = ChCl-glycerol (40 °C) A2 = ChCl-lactic (40 °C), A3 = ChCl-malic (40 °C), A4 = ChCl-malonic (40 °C, A5 = ChCl-glycerol (50 °C), A6 = ChCl-lactic (50 °C), A7 = ChCl-malic (50 °C) and A8 = ChCl-malonic (50 °C).

malonic acid being the highest. Relative to the alkaline control which was 91.4 °C, most of the NADES conditions yielded higher thermal stability. Alkaline controls denature at 94.37 °C (uncooked) and 91.4 °C (cooked), generally below the best NADES conditions. A recent report on brewer's spent-grain protein showed that NADES-extracted material denatured at 81 °C, compared with 68 °C for the alkaline-extracted control, which is similar to this study's results.[34]

Complementary to FTIR, Differential Scanning Calorimetry (DSC) was used to evaluate the thermal stability of the extracted proteins. DSC provides information on the denaturation temperature and enthalpy change (ΔH) associated with the unfolding of protein structures.[92] These parameters are direct indicators of molecular integrity and resistance to thermal degradation. This DSC thermograms were used to assess the thermal stability of crab shell proteins extracted by cooked and uncooked crab shells under different extraction conditions. Fig. 16 shows the DSC thermograms of uncooked crab shells proteins extracted with different NADES. Choline chloride with glycerol, lactic, malic, or malonic acids at 40 °C and 50 °C temperature was used for the extraction and compared with an alkaline extract as the control. All samples showed a single dominant endotherm whose peak temperature (denaturation temperature) varied with solvent composition and extraction temperature. At 40 °C extraction, denaturation temperatures were 105.5–121.9 °C in all types of NADES, ChCl-malonic acid being the highest stable proteins. Raising the extraction temperature to 50 °C the denaturation temperature range shifted downward between 100.4 °C and 111.7 °C, but ChCl-malonic acid remained the highest stable proteins. In terms of thermal stability of proteins ranking remained malonic > malic > glycerol. Lactic acid showed almost similar thermal stability to glycerol.

However, all NADES extracts exhibited higher denaturation temperature than the alkaline extract which was 94.37 °C, indicating greater thermal stability than the alkaline control under the NADES conditions. Across the same NADES and extraction temperatures, uncooked extracts denatured at higher temperature (100–122 °C) than the denaturation temperature of cooked extracts (85–100 °C). This pattern is consistent with thermal processing that cooked crab shells has undergone. Pre-cooking partially unfolds and aggregates shell proteins, lowering the thermal stability and reducing the denaturation temperature in the DSC thermogram. In marine crab muscle, DSC typically shows two cooperative transitions. The first one is due to myosin around 49–52 °C and second one due to actin around 77–78 °C.[93] Compared to these values the denaturation temperature values of both cooked and uncooked shells are higher according to the results from this study. Even after subjecting to the heat processing, crab shell proteins have higher thermal stability than muscle proteins.

A recent study on abalone shells showed that shell-embedded proteins can persist even after toasting at 200–300 °C for 10 minutes. Proteomics still identified dozens of proteins at 300 °C due to the resilience to destruction of mineral/organic interfaces and nanopore formation during heating, which helps protect intracrystalline proteins which is bound together with minerals in the matrix.[94] This mechanism aligns very well with this study as crab shell proteins are bound with chitin and minerals in the shell structure, which limits proteins exposure for denaturation by heat (in the cooked crab shell protein). There are no peer-reviewed DSC studies that report denaturation temperatures for purified crab-shell protein isolates. This study therefore fills a clear gap by providing DSC-derived thermal stability data for shell proteins extracted with multiple NADES at two extraction temperatures and thermally processed (uncooked *vs.* cooked), and by comparing these against an alkaline-extracted control.

5 Conclusions

This study is about biorefinery route for snow crab shells that separates minerals by demineralization, fat and pigments removal, and protein recovery by solubilization and precipitation. Using a mild citric-acid demineralization (1 : 20 w/v) achieved the highest mineral removal in both cooked and uncooked shells. Critically, while NADES are widely applied to extract chitin where a combined demineralization and deproteinization process produces high-purity chitin, this study targets high-purity proteins as a co-product by first demineralizing and then extracting proteins. In this study, ChCl-malic gave the highest extraction yield (uncooked 12.64% at 1 : 30/50 °C) and performed comparably to the alkaline control (13.79% ± 0.85) for uncooked shells. For cooked shells, ChCl-malic and ChCl-malonic were top performers but remained just below the conventional method (14.71% ± 0.68), underscoring a small yield gap that is acceptable given the sustainability advantages of NADES. In terms of protein concentration of the uncooked shells isolates, ChCl-malonic (1 : 30/50 °C) was the highest, followed by ChCl-malic, then ChCl-lactic, with ChCl-glycerol being the least effective in both the cooked and uncooked crab shells. Overall, this two-step, NADES-assisted approach limits usage of harsh chemicals, recovers protein that would otherwise be lost in chitin–extraction processes, and utilize crab shells to their maximum potential. NADES extraction yielded proteins with improved functionality compared to alkaline extraction from both cooked and uncooked crab shell proteins. Among solvents, ChCl-malonic gave the most balanced profile: highest solubility at pH 3,5,7 in uncooked shells, highest FC/FS at pH 5, strong ESI at pH 3, lighter color (high L^*), and highest DSC stability in both cooked and uncooked proteins. Secondly, ChCl-malic also showed improved functionality. ChCl-glycerol tended to have comparatively lower functionality and ChCl-lactic was intermediate. Cooked proteins were more soluble compared to uncooked proteins with all NADES. However, cooked proteins were less heat-stable than uncooked proteins. FTIR changes were minimal between 40–50 °C. Identifying the functional potential of these protein isolates highlights their suitability for food applications, transforming crab shell waste into a valuable zero-waste resource with utilization potential across all its components. Considering the outcomes of this study, several directions are recommended to advance the scientific understanding and practical application of crab shell protein valorization. Future research should focus on allergenicity and food safety of proteins extracted from crab shells. As crustacean proteins are known allergens, it is vital to evaluate whether the extraction processes (NADES or alkaline) alter the allergenicity, reduce, or retain allergenic potential. Detailed *in vitro* allergenicity assessments, coupled with protein digestibility studies, should be conducted to ensure the safe inclusion of crab shell-derived proteins in food systems. This will be particularly important if the isolates are intended for functional or therapeutic food applications.

Conflicts of interest

The authors, Tharuka Wijesekara, and Idaresit Ekaette declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

Data availability

Data for this article, including the design of NADES are available within this article.

Supplementary information (SI): rough cost estimates for the recovery of NADES per cycle of crab protein extraction. See DOI: https://doi.org/10.1039/d6fb00024j.

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