Research Article

Extraction of Chitin from Giant Tiger Prawn (*Penaeus monodon*) Shrimp Shell Using Deep Eutectic Solvents and Citric Acid

Tran Chi Hai, Nguyen Tan Dat, Quach Le Anh Tuan, Huynh Le Thanh Ngan, Nguyen Duong Thien Tu, Nguyen Tan Thinh, Duy Nhat Quang and Le Thi Hong Anh*
Ho Chi Minh City University of Industry and Trade, Ho Chi Minh City, Vietnam

Phan Van Man*
Ba Ria – Vung Tau College of Technology, Vung Tau City, Vietnam

* Corresponding author. E-mail: anhlth@hufi.edu.vn, pvman.dbv@moet.edu.vn DOI: 10.14416/j.asep.2023.10.006
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Abstract
Chitin, a natural biomass resource, has shown great promise for a wide range of applications because of its high bioactivity. This study evaluated the effectiveness of deep eutectic solvents (DESs) mixed with citric acid as a method for extracting chitin from the shells of giant tiger prawn shrimps (*Penaeus monodon*). The purity and physicochemical properties of the extracted chitin were compared with those obtained using the traditional chemical extraction method and commercial chitin. The results showed that the highest chitin purity (99.22%) was achieved when choline chloride-glycerol (ChCl–Gl) was used in a 1:2 molar ratio with a citric acid content of 5% w/v (CG2-5%). Additionally, the extracted chitin had a molecular weight of 3.75×10^5 Da and a crystallinity index of 81.34%, which was slightly higher than that of chitin extracted using the conventional method (3.24×10^5 Da and 73.59%). However, there was no significant difference between chitin extracted by CG2-5% and commercial chitin. This suggests that the structure of the biopolymer remained intact following the CG2-5% extraction process. The α-chitins in tiger prawn shrimp shells, as confirmed by Scanning Electron Microscopy (SEM), Fourier Transform Infrared (FTIR) spectroscopy, and X-ray Diffraction Analysis (XRD), are analogous to commercial shrimp chitin. These results, achieved without employing potentially harmful chemicals, demonstrate that CG2-5% can efficiently enhance chitin extraction from diverse raw biomass sources without jeopardizing the polymer's structural stability.

Keywords: Chitin, Deep eutectic solvents, Extraction, Giant tiger prawn

1 Introduction

Chitin, consisting of β-(1-4) linked N-acetylglucosamine residues, is the second most plentiful biopolymer found in nature, after cellulose [1]–[3]. It is primarily sourced from the shells of crustaceans, such as shrimp and crabs [4]–[6]. Chitin and its by-products have found extensive application in the food, cosmetic, and pharmaceutical sectors due to their various advantages such as being biodegradable, biocompatible, non-toxic, barely immunogenic, and thermally stable [7], [8]. Annually, the global seafood industry discards approximately 6–8 million tons of crustacean shell waste, with shrimp accounting for around 50–70% of this waste. Consequently, the shrimp processing sector produces massive quantities of by-products, such as shrimp heads and carapaces, which are typically utilized for low-value animal feed and biofertilizers. Inappropriate disposal of these crustacean shells can result in significant environmental problems, including unpleasant odors and mineral sedimentation in landfills. Thus, converting these by-products into high-value products and addressing these environmental concerns is of utmost importance.

Prior research has revealed that shrimp shells are primarily composed of chitin (15–40%), protein (20–40%), and calcium and magnesium carbonate (20–50%). They also contain smaller amounts of lipids, astaxanthin, and other minerals [9], [10]. Thus, to generate high-quality chitin, removal of other components, including proteins, lipids, and assorted impurities, is required. In current industrial processing, chitin extraction is carried out by two different methods: biological and chemical processes. Both methods involve two primary stages: demineralization and deproteinization [11], [12]. Biological techniques offer numerous benefits over chemical methods in terms of environmental friendliness and sustainability. However, the production of chitin on a commercial scale remains inefficient and costly because of the complexity of the chitin production process and the lack of efficient fermentation methods [11], [13]. Chemical extraction of chitin requires strong acids and alkalis to eliminate minerals and proteins, which generates substantial volumes of corrosive wastewater that are detrimental to the environment [5], [8], [11], [12].

Recently, chitin extraction using deep eutectic solvents (DESs) from crustacean shells has been reported as an alternative method [11], [12]. DESs show potential as chitin production solvents owing to their numerous benefits, such as low toxicity, affordability, simple synthesis, biodegradability, and minimal volatility [11]–[14]. Saravana *et al.*, used choline chloride and citric acid as a DES, achieving chitin with low ash content (1.18%) but high protein content (8.37%) [15]. Xie *et al.*, also applied a DES mixture of choline chloride and citric acid to extract chitin from lobster shells, but the obtained chitin exhibited high ash (5.0%) and protein (4.1%) contents compared with those extracted via acid/alkali methods [16]. In addition, DESs containing citric acid as a hydrogen bond donor require a high acid concentration to extract chitin (not less than 50% molar ratio), which leads to high equipment demands [13], [17]. To the best of our knowledge, there have been no previous studies on chitin extraction using a combination of citric acid, choline chloride with glycerol, or choline chloride with urea. Thus, the objective of this study was to identify a combination of choline chloride, glycerol, choline chloride, and urea in various molar proportions, supplemented with minimal amounts of citric acid, that could be used to extract high-quality chitin from giant tiger prawn shrimp shells (*Penaeus monodon*). The chemical compositions and molecular weights of the chitin samples were analyzed. Furthermore, the physicochemical structure of the isolated chitin was compared to that of both traditionally extracted and commercially available chitin. This comparison was facilitated by X-ray diffraction, Fourier-transform infrared spectroscopy, and scanning electron microscopy.

2 Experiments

2.1 *Materials and chemicals*

Waste from Giant tiger prawn (*Penaeus monodon*) shrimp, including the head and carapace, was sourced from Minh Phu Ltd., located in the Ca Mau Province, Vietnam. The waste was cleaned with tap water prior to being dried in a vacuum oven at 60 °C for a day [13]. The dried shrimp waste was ground into a fine powder with a particle size of 100 μm by using a grinder and stored in plastic bags.

Choline chloride (ChCl), as a hydrogen bond acceptor (HBA), as well as glycerol (Gl) and urea (Ur), as hydrogen bond donors, were purchased from Sigma-Aldrich (St. Louis, MO, USA) for the production of deep eutectic solvents (DES). Sinopharm Chemical Reagent Co. Ltd. (Shanghai, China) was the supplier of commercial citric acid and chitin. All the chemicals utilized were of analytical grade.

2.2 *Effects of different DESs on chitin extraction*

The DESs were prepared by mixing ChlCl with Gl or Ur in varying molar ratios, as detailed in Table 1. The DES mixtures were heated in a thermostatic bath at 90 °C according to a previous study [18]. The DESs were stirred until the mixtures formed a clear solution and were evaluated using a CCD camera. The viscosity of each DES was examined using a DVII-Brookfield viscometer from Middleboro, USA, utilizing an S52 adapter. The examination was conducted under a regulated temperature of 35 ± 2 °C, rotating at a speed of 50 rpm for a duration of 10 s at 24 °C.

In order to extract chitin, 1.0 g of powdered shrimp residue was mixed with 20.0 mL of DES solvent and magnetically stirred at 90 °C for 3 h [19]. Following this, the mixture was centrifuged using

an SL-706 centrifuge (Solab, Piracicaba, Brazil) at a speed of 6,000 rpm for 5 min, and the precipitate was recovered. The recovered residue was subsequently decolorized with a 1:2:4 mixture of chloroform, methanol, and distilled water at room temperature for 30 min and then dried at 60 °C for 24 h to yield the final chitin product [2]. The physicochemical structure of the isolated chitin was analyzed by XRD, SEM, and FTIR. The DES system that provided the purest chitin was selected for the subsequent experiment.

2.3 *Effects of different citric acid concentrations in DES on chitin extraction*

Various amounts of citric acid were introduced into the DES system, and the mixtures were continuously stirred for 2 h at precise temperatures, as indicated in Table 2. The extraction process was implemented according to the methodology detailed by Sun *et al.*, [19]. The powder from the shrimp shell was combined with a DES system that contained varying concentrations of citric acid and was then extracted at 90 °C for 3 h. After that, the mixed reaction was cooled and filtered using a Buchner funnel. The collected solid was rinsed with distilled water to obtain a neutral filtrate. The acquired residue was then decolorized using 1:2:4 ratios of chloroform, methanol and distilled water. The decolorized materials were then stored in a hot-air oven at 60 °C for a period of 24 h to yield the dried chitin product [2]. Each trial was conducted in triplicates. In order to draw a comparison with the samples produced through the co-solvent DES, chitin was similarly extracted from shrimp shells utilizing both DES and traditional chemical techniques. The proportion of chitin (%) was determined employing the subsequent Equation (1):

$$\textit{Chitin content}\ (\%) = \textit{Dried chitin extracted}\ (g) \times 100 / \textit{Raw material}\ (g) \quad (1)$$

2.4 *Traditional extraction of chitin*

The chemical extraction of chitin predominantly consists of two crucial steps: the demineralization and deproteinization of crustacean shells [2]. Initially, the shrimp powder (5.0 g) was demineralized using HCl (2 M) at 60 °C for 150 min with continuous stirring. The substance was then rinsed with distilled water until it reached a neutral pH. After that, the substance was deproteinized using a 10% NaOH solution (50 mL). The mixture was heated and persistently stirred at 90 °C for 160 min. Subsequently, the sample was subjected to decolorization, followed by a rinse with distilled water and oven drying at 100 °C to produce the purified chitin.

2.5 *Measurement of chitin purity*

The moisture content was measured by drying it for 24 h at 105 °C, immediately followed by placing it in a desiccator for half an hour to cool down to room temperature. The water content of the sample was determined by considering the rate of weight loss before and after drying.

The ash content was ascertained by utilizing a muffle furnace at 600 °C for 240 min [20]. The ash content was determined by comparing the ash residue's proportion to the original weight of the sample. The Bradford method was used to measure the residual protein content [21]. The purity of chitin [Equation (2)] was calculated as described by Morgan *et al.* [17] and Feng *et al.* [22].

Table 1: The purity, yield, ash, and protein content of chitin extracted with different deep eutectic solvents

Deep Eutectic Solvent	Sample Code	Molar Ratio (HBA: HBD)	Viscosity (Pa.s)	Chitin Yield (%)	Chitin Purity (%)	Ash (%)	Protein (%)
ChCl-Gl	CG1	1:1	0.19	$22.49^{b} \pm 0.91$	$87.58^{c} \pm 1.21$	$7.97^{a} \pm 0.62$	$4.45^{a} \pm 0.21$
	CG2	1:2	0.11	$20.89^{c} \pm 0.89$	$92.90^{ab} \pm 1.84$	$4.01^{b} \pm 0.49$	$3.09^{b} \pm 0.45$
	CG3	1:3	0.09	$21.10^{bc} \pm 0.81$	$88.54^{c} \pm 1.75$	$6.79^{a} \pm 0.57$	$3.67^{b} \pm 0.60$
ChCl-Ur	CU1	1:1	0.21	$25.46^{a} \pm 0.81$	$86.88^{c} \pm 1.55$	$8.61^{a} \pm 0.48$	$4.51^{a} \pm 0.47$
	CU2	1:2	0.18	$22.53^{b} \pm 0.72$	$90.80^{b} \pm 1.67$	$5.19^{b} \pm 0.52$	$4.01^{a} \pm 0.45$
	CU3	1:3	0.11	$24.01^{a} \pm 0.81$	$86.30^{c} \pm 1.14$	$7.75^{a} \pm 0.41$	$5.95^{a} \pm 0.51$
Chemical extraction	CE	-	1.10	$15.63^{d} \pm 0.83$	$98.16^{a} \pm 1.35$	$0.55^{c} \pm 0.04$	$1.29^{c} \pm 0.35$

Note: In each column, distinct lowercase letters attached to values denote a significant difference at p-value < 0.05.

Table 2: The purity, yield, ash, and protein content of chitin, extracted using deep eutectic solvent and citric acid

Deep Eutectic Solvent	Citric Acid Content (%w/v)	Sample Code	Viscosity (Pa.s)	Chitin Yield (%)	Chitin Purity (%)	Ash (%)	Protein (%)
ChCl-Gl	0	CG2–0.0%	0.11	$20.93^{b} \pm 0.97$	$92.08^{b} \pm 2.01$	$4.03^{b} \pm 0.08$	$3.89^{a} \pm 0.05$
	2.5	CG2–2.5%	0.12	$20.03^{b} \pm 0.81$	$93.09^{b} \pm 2.12$	$4.95^{b} \pm 0.06$	$1.96^{c} \pm 0.09$
	5	CG2–5.0%	0.12	$19.01^{c} \pm 0.71$	$99.00^{a} \pm 1.85$	$0.05^{d} \pm 0.01$	$0.95^{d} \pm 0.09$
	7.5	CG2–7.5%	0.20	$20.98^{b} \pm 0.92$	$93.40^{b} \pm 1.98$	$2.05^{c} \pm 0.03$	$4.55^{a} \pm 0.08$
	10	CG2–10%	0.25	$21.11^{b} \pm 0.95$	$92.14^{b} \pm 2.04$	$2.97^{c} \pm 0.08$	$4.89^{a} \pm 0.10$
ChCl-Ur	0	CU2–0.0%	0.18	$23.53^{a} \pm 1.05$	$90.12^{c} \pm 1.89$	$6.51^{a} \pm 0.08$	$4.37^{a} \pm 0.09$
	2.5	CU2–2.5%	0.19	$23.09^{a} \pm 1.01$	$91.18^{bc} \pm 2.01$	$3.50^{c} \pm 0.06$	$2.32^{b} \pm 0.08$
	5	CU2–5.0%	0.20	$22.09^{bc} \pm 0.93$	$93.96^{b} \pm 1.89$	$1.50^{d} \pm 0.09$	$2.54^{b} \pm 0.10$
	7.5	CU2–7.5%	0.25	$21.32^{b} \pm 0.91$	$91.46^{bc} \pm 1.84$	$1.95^{d} \pm 0.08$	$2.59^{b} \pm 0.09$
	10	CU2–10%	0.28	$23.32^{a} \pm 1.05$	$89.14^{c} \pm 2.02$	$2.13^{c} \pm 0.10$	$2.73^{b} \pm 0.11$
Chemical extraction	-	CE	0.11	$15.63^{d} \pm 0.90$	$98.49^{a} \pm 2.11$	$0.19^{e} \pm 0.09$	$1.32^{c} \pm 0.12$

Note: In each column, distinct lowercase letters attached to values denote a significant difference at p-value < 0.05.

$$Purity\ (\%) = 100 - ash\ content\ (\%) - protein\ content\ (\%) \quad (2)$$

2.6 *Scanning electron morphology (SEM)*

The morphological structure of the chitin obtained was determined using a scanning electron microscope (SEM) (Quanta 650-FEG, FEI, USA). The samples (raw material, extracted chitin, and commercial shrimp chitin) were dehydrated, secured to sticky tape, and layered with gold. Images were captured using a voltage of 5 kV and a magnification level of 5,000.

2.7 *Fourier transform infrared spectroscopy (FTIR)*

The FTIR analysis was conducted using a Nicolet 6700 spectrophotometer (Thermo Fisher Scientific, Massachusetts, USA), which was adjusted for 32 scans and a resolution of 4 cm^{-1}. The analysis of the compounds' molecular structures was conducted with the spectral range set from 4000–400 cm^{-1}, at a 4 cm^{-1} resolution.

The degree of deacetylation (AD) [Equation (3)] of chitin samples were calculated by FTIR spectra as described by Zhang *et al.* [13].

$$AD\ (\%) = A_{1650}/A_{3450} \times 115\% \quad (3)$$

The absorbance at 1650 and 3450 cm^{-1} are represented by A_{1650} and A_{3450}, respectively.

2.8 *Molecular weight determination of chitin samples*

The chitin's molecular weight (Mw) was assessed at 30 °C, utilizing an Ubbelohde viscometer (Reagecon, Calibre Scientific Co., Ltd., Germany). Chitin was dissolved in N, N, N-dimethylacetamide (DMAc) solution containing 5 wt% LiCl. Mw was calculated using the Mark–Houwink–Sakurada Equation (4) [23].

$$[\eta] = KM_w\alpha \quad (4)$$

where intrinsic viscosity is represented by $[\eta]$, the constant K equals 7.6×10^{-5} dL/g, and α is equal to 0.95.

2.9 *X-ray Diffraction*

Powder X-ray diffraction (XRD) (D8 Advance, Bruker, Billerica, MA, USA) analysis of chitin was performed using a D8 Advance X-ray diffractometer. Data were collected at a scan rate of 1°/min at a scan angle of 5–40°. The crystallinity indexes (CrI) were calculated using the following Equation (5) [2], [15]:

$$CrI\ (\%) = [I_{10} - I_{am}] \times 100/I_{10} \quad (5)$$

The maximum intensity of the crystalline region at 20° is denoted as I_{10}, while the maximum intensity of the amorphous diffraction at 16° is represented as I_{am}.

2.10 *Statistical analysis*

The experimental design was statistically analyzed and subjected to ANOVA using version 25 of the SPSS Statistics software. All determinations were conducted in triplicate. All data are expressed as mean ± SD, using a significance level of p-value <0.05.

3 Results and Discussion

3.1 *Effects of different DESs on chitin extraction*

The chitin yields and purities obtained using the different DESs were analyzed and compared with those obtained using CE, as depicted in Table 1. The results showed that the quantity of chitin extracted fluctuated from 86.42–98.11 mg/g dm, based on the specific DESs used. The maximum chitin extraction was achieved with ChCl:Ur (25.46%) at a 1:1 molar ratio. These yields surpassed those obtained using the conventional acid/alkali method by 1.47 times. However, chitin yield was not the primary factor considered during chitin extraction and isolation. Protein and ash content serve as essential indicators to confirm the purity of chitin [2], [15].

Therefore, this study disregarded the yield and selected a DES solvent that could reduce the protein and ash contents in the extracted chitin. As illustrated in Table 1, among the two DES systems examined at different HBA: HBD molar ratios, CG2 demonstrated the lowest levels of ash (4.01%) and protein content (3.09%), followed by CU2 (5.19% and 4.01%), respectively. This might be attributed to the high reactivity of ChCl due to its chlorine presence, while Gl acts as a potent disruptor of hydrogen bonds [4], [11]. When these substances come into contact with chitin, they can effectively break down its molecular structure, leading to the destruction of the chemical bonds [4], [13]. Additionally, urea (Ur) has the capacity to interrupt the hydrogen bonds and disulfide bridges in chitin, thus causing its degradation [4]. However, according to our findings, chitin produced from CG2 and CU2 still contained more ash and protein than chitin obtained through acid/alkali methods (0.55% and 1.29%, respectively). This observation is consistent with that of Saravana *et al.* [15], who noted that conventional extraction techniques can effectively remove the majority of minerals and proteins from crustacean wastes [14]. Nonetheless, the main challenges in acidic/alkali chitin extraction processes are various environmental issues [5], [8], [10]. According to Zhao *et al.* [4], citric acid is sufficient to react with mineral salts, especially calcium carbonate, in shrimp shells, thus improving chitin purity by enhancing the removal of protein and mineral salts. However, the combination of DESs and citric acid for chitin extraction is still limited. Therefore, CG2 and CU2 combined with citric acid were used to improve the efficiency of protein and ash removal from the extracted chitin (Table 2).

3.2 *Effects of different citric acid concentrations on chitin extraction*

Table 2 displays the effect of different citric acid concentrations on the yield and purity of chitin. The findings showed that when the concentration of citric acid was increased from 0% (w/v) to 5.0% (w/v), there was a decline in the chitin yield from 23.53% to 19.01%. In contrast, the purity of the chitin rose from 93.80% to 99.00%. The highest purity was attained at CG2 with 5% citric acid (CG2–5%), which was slightly higher than the acid/alkali process (p-value < 0.05). The reason might be that citric acid assists the deep eutectic solvent in disrupting the complex network among chitin, organic mineral salts, and cell membrane proteins [4]. Consequently, proteins and mineral salts were dispersed more readily within the extraction solvent, enabling easier separation from the chitin residue. Additionally, the viscosity of the CG2–5% was lower than that of other extraction solvents, which may make it easier to penetrate cell walls, thus increasing extra-molecular extraction and improving the purity of the extracted chitin [24]. However, chitin purity decreased slightly when citric acid concentration increased from 7.5% to 10.0% (w/v). The decrease may be due to the increased quantity of citric acid, which can potentially influence the strength of hydrogen bonds in CG2 and potentially disrupt the intermolecular interactions of solutes in CG2 with mineral salts and shrimp shell powder proteins. Furthermore, a higher concentration of citric acid in CG2 might lead to a higher level of viscosity, obstructing the solvent's penetration into the extraction matrix, and resulting in low purity of extracted chitin [4]. Similar findings have been observed for chitin

extracted from tiger shrimp waste using ChCl-Gl and acetic acid [13]. In contrast, the purity of chitin extracted using CU2 (ChCl-Ur) combined with citric acid did not show a significant improvement (p-value < 0.05). The findings suggest that the combination of CG2 and 5% citric acid is a successful technique for obtaining high-purity chitin from shrimp shells.

3.3 *Effect of extraction conditions on chitin*

The effects of different factors including extraction temperature, time, and solid-to-solvent ratio on the yield and purity of chitin extracted from the shells of black tiger shrimp (*Penaeus monodon*) were examined. Table 3 demonstrates that the purity of chitin notably increased from 86.83–99.00%, while the yield gradually dropped from 26.93–20.18% as the extraction temperature rose from 40–80 °C. Similarly, in Table 4, an increase in the extraction time from 1–4 h resulted in an increase in chitin purity from 89.31–99.21%, and a corresponding decrease in yield from 28.90–20.01%. Higher temperatures and extended extraction periods enhance molecular mobility, facilitating faster diffusion of extracted molecules into the solvent [13], [23]. However, a slight decline in chitin purity was observed when the temperature surpassed 80 °C and the extraction time was lengthened to 5 h. This might be attributed to the escalation in extraction temperature and the fact that a lengthier extraction time increases the volatility of water in the extraction solvent. This subsequently boosts the viscosity and minimizes the diffusivity of the protein and mineral salts to be extracted in DESs [24], [25]. Furthermore, the elevated temperature and prolonged processing duration could result in a weakened hydrogen bond in CG2 with 5% citric acid. This can potentially reduce the dissolution of protein and mineral salts in the solvent, thereby lowering the purity of the extracted chitin. Hence, the optimal temperature and duration for chitin extraction were determined to be 80 °C and 4 h. These parameters were then applied in the following experiment.

Table 3: Effect of temperature process on the purity, yield, ash, and protein content of chitin

Deep Eutectic Solvent	Sample Code	Viscosity (Pa.s)	Temperature Process (°C)	Chitin Yield (%)	Chitin Purity (%)	Ash (%)	Protein (%)
ChCl-Gl[(2)]	CG2–5.0%	0.21	40	$26.93^{a} \pm 0.81$	$86.83^{d} \pm 2.13$	$7.99^{a} \pm 0.19$	$5.18^{a} \pm 0.48$
		0.19	50	$24.11^{b} \pm 0.95$	$89.28^{c} \pm 2.15$	$6.78^{ab} \pm 0.21$	$3.94^{b} \pm 0.20$
		0.10	60	$22.94^{c} \pm 0.51$	$91.65^{c} \pm 2.01$	$5.54^{b} \pm 0.51$	$2.81^{b} \pm 0.35$
		0.09	70	$21.91^{cd} \pm 0.67$	$96.43^{b} \pm 1.91$	$1.53^{c} \pm 0.41$	$2.04^{b} \pm 0.31$
		0.08	80	$20.18^{d} \pm 0.22$	$99.00^{a} \pm 2.00$	$0.05^{d} \pm 0.01$	$0.95^{d} \pm 0.05$
		0.12	90	$21.91^{cd} \pm 0.79$	$97.98^{b} \pm 1.95$	$1.23^{c} \pm 0.34$	$0.79^{b} \pm 0.05$
		0.12	100	$22.01^{c} \pm 0.55$	$96.08^{b} \pm 1.14$	$2.60^{c} \pm 0.36$	$1.25^{c} \pm 0.02$
Chemical extraction[(1)]	CE	1.10	-	$15.63^{d} \pm 0.76$	$98.09^{ab} \pm 2.01$	$0.59^{d} \pm 0.02$	$1.32^{c} \pm 0.04$

Note: [(1)] The alkali pretreatment involves a 10% NaOH solution at 90 °C for 3 h, followed by an acid treatment for 2.5 h at room temperature [2].
[(2)] DES extraction occurs under conditions of a solid to solvent ratio of 1:20 for 3 h [16]. Different lowercase letters in each column signify a significant variation at p-value < 0.05.

Table 4: Effect of extraction time on the purity, yield, ash, and protein content of chitin

Deep Eutectic Solvent	Sample Code	Viscosity (Pa.s)	Time Process (h)	Chitin Yield (%)	Chitin Purity (%)	Ash (%)	Protein (%)
ChCl-Gl[(2)]	CG2–5.0%	0.09	1	$28.90^{a} \pm 1.32$	$89.31^{d} \pm 1.01$	$6.51^{a} \pm 0.61$	$4.18^{a} \pm 1.00$
		0.09	2	$26.19^{b} \pm 1.45$	$94.13^{c} \pm 1.12$	$2.93^{b} \pm 0.05$	$2.94^{b} \pm 0.41$
		0.08	3	$21.14^{c} \pm 1.13$	$96.97^{b} \pm 1.03$	$1.02^{c} \pm 0.02$	$2.01^{b} \pm 0.11$
		0.12	4	$20.01^{c} \pm 1.35$	$99.21^{a} \pm 2.01$	$0.05^{c} \pm 0.01$	$0.74^{d} \pm 0.05$
		0.15	5	$21.18^{c} \pm 1.31$	$98.91^{ab} \pm 1.46$	$0.05^{c} \pm 0.01$	$1.22^{c} \pm 0.04$
Chemical extraction[(1)]	CE	1.01	-	$15.63^{d} \pm 1.64$	$98.09^{ab} \pm 1.57$	$0.59^{d} \pm 0.02$	$1.32^{c} \pm 0.04$

Note: [((1))] The alkali pretreatment involves a 10% NaOH solution at 90 °C for 3 h, followed by an acid treatment for 2.5 h at room temperature [2].
[(2)] DES extraction takes place under conditions of a solid-to-solvent ratio of 1:20 [16] at an optimal extraction temperature of 80 °C. Different lowercase letters in each column signify a significant variation at p-value < 0.05.

As shown in Table 5, the ratio of shrimp shell powder to the extraction solvent significantly affected chitin yield and purity. When the mass ratio of shrimp shell powder to extraction solvent was varied from 1:5 to 1:25, with all other conditions constant, the purity increased from 89.31 to 99.22% and the chitin yield decreased from 26.56 to 19.75%. A higher solid-liquid ratio increases the diffusion of components into solvents and accelerates mass transfer, thus improving the chitin purity [19]. However, increasing the solid-liquid ratio from 1:20 to 1:25 resulted in a slight reduction in chitin purity (p-value < 0.05). To achieve a balance between high purity and use of the least amount of the deep eutectic solvent (CG2; 5%), the solid-to-solvent ratio should be 1:20 w/v to avoid unnecessary waste.

Therefore, the optimal extraction of chitin by CG2 using 5% citric acid was achieved at 80 °C for 4 h, utilizing a solid-to-solvent ratio of 1:20 w/v. Under these conditions, the chitin yield and purity amounted to 19.75% and 99.22%, respectively. The chitin yield and purity obtained were 1.2–1.4 times higher than those attained with the CE method.

3.4 *Analysis of the chemical composition of the extracted chitin*

The chemical components of the chitin product, selected under ideal conditions, were analyzed and compared with those of shrimp shells, CE chitin, and commercially available chitin (Table 6). The ash content in shrimp shells accounted for 43.15%, but after treatment with CG2–5%, it decreased to 0.03%. This indicated that the minerals from the raw shrimp shell materials were thoroughly removed, consistent with FTIR results (Figure 1) [13], [17]. In addition, protein and another important component in shrimp shells were present at approximately 13.91% and reduced to 0.75% after CG2–5% treatment, even slightly lower than 0.75% of the protein in commercial chitin. Notably, the purity of both commercially available chitin and chitin extracted from shrimp shells treated with 5% citric acid in CG2 exceeded 99.22%.

3.5 *Structural characterization of the extracted chitin*

The chitin obtained (CG2–5% and CE) was examined using FTIR, XRD, and SEM, and was subsequently contrasted with commercially available shrimp chitin. Figure 1 illustrates the FTIR spectra for the extracted chitin, commercial shrimp chitin, and shrimp shells. In the diagram, the absorption peak at 874 cm^{-1}, which is present in the raw materials, almost completely disappeared in the extracted and commercial shrimp

Table 5: Effect of solid-to-liquid ratio on the purity, yield, ash, and protein content of chitin

Deep Eutectic Solvent	Sample Code	Viscosity (Pa.s)	Solid to Solvent Ratio (w/v)	Chitin Yield (%)	Chitin Purity (%)	Ash (%)	Protein (%)
ChCl-Gl[(2)]	CG2–5.0%	0.25	1:5	$26.56^{a} \pm 0.36$	$87.23^{d} \pm 1.85$	$7.99^{a} \pm 0.54$	$4.78^{a} \pm 0.14$
		0.18	1:10	$23.11^{b} \pm 0.38$	$93.11^{c} \pm 1.94$	$3.98^{b} \pm 0.02$	$2.91^{b} \pm 0.22$
		0.12	1:15	$22.94^{b} \pm 0.44$	$95.02^{c} \pm 2.03$	$2.54^{b} \pm 0.21$	$1.44^{c} \pm 0.03$
		0.10	1:20	$20.91^{c} \pm 0.61$	$99.22^{a} \pm 2.04$	$0.03^{d} \pm 0.00$	$0.75^{d} \pm 0.01$
		0.09	1:25	$21.05^{c} \pm 0.52$	$94.23^{c} \pm 1.94$	$3.63^{b} \pm 0.07$	$2.14^{b} \pm 0.03$
Chemical extraction[(1)]	CE	0.87	-	$16.99^{d} \pm 0.34$	$98.48^{b} \pm 1.89$	$0.54^{c} \pm 0.02$	$0.98^{d} \pm 0.04$

Note: [(1)] The alkali pretreatment involves a 10% NaOH solution at 90 °C for 3 h, followed by an acid treatment for 2.5 h at room temperature [2].

[(2)] The DES extraction takes place under conditions with an optimal extraction temperature and time (80 °C and 4 h). Different lowercase letters in each column signify a significant variation at *p*-value < 0.05.

Table 6: Components of shrimp shell, commercially available chitin, and produced chitin

Sample Code	Chitin Purity (%)	Ash (%)	Protein (%)	Moisture Content (%)
Raw material	-	$43.15^{a} \pm 1.02$	$13.91^{a} \pm 0.71$	$5.85^{a} \pm 0.25$
CE	98.48ab ± 1.36	$0.54^{b} \pm 0.02$	$0.98^{b} \pm 0.04$	$2.15^{b} \pm 0.10$
CG2–5%	99.22a ± 2.04	$0.03^{c} \pm 0.00$	$0.75^{c} \pm 0.01$	$2.12^{b} \pm 0.09$
Commercial chitin	99.25a ± 1.34	$0.03^{c} \pm 0.01$	$0.74^{c} \pm 0.05$	$2.05^{b} \pm 0.12$

Note: Values with different lowercase letters in each column indicate a significant difference at *p*-value < 0.05.

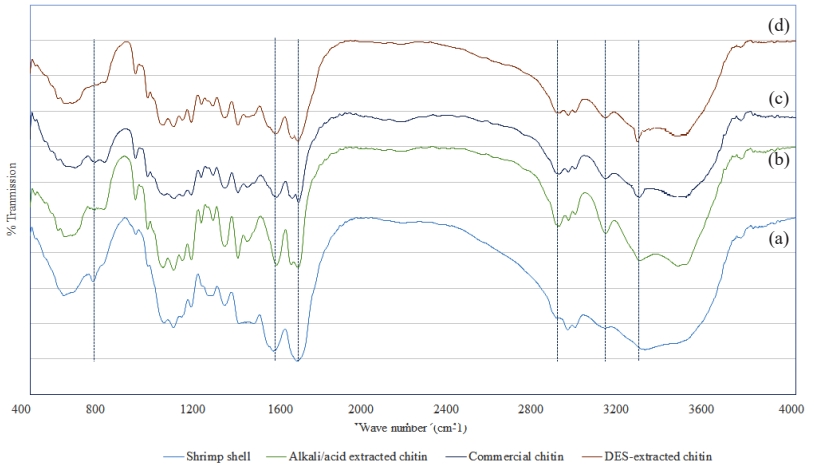

Figure 1: FTIR spectra of (a) raw material, (b) CE chitin, (c) CG2–5% chitin, and (d) commercial shrimp chitin.

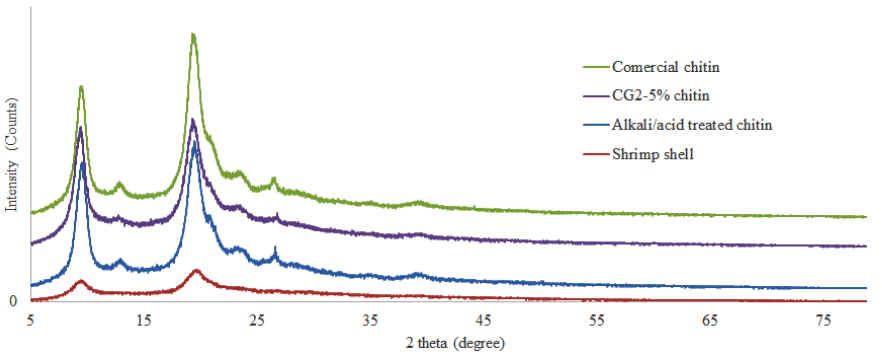

Figure 2: X-ray diffractograms of raw material, CE chitin, CG2–5% chitin, and commercial shrimp chitin.

chitin. This reduction in peak intensity in the extracted chitin resulted from the partial elimination of mineral salts, specifically $CaCO_3$, in shrimp cells, which is consistent with the results of Sun *et al.* [19] and Bisht *et al.* [25]. This research additionally highlighted two peaks at 1650 and 1621 cm^{-1}, derived from C=O-stretching vibrations (amide I). These are distinguishing bands of α-chitin [2], [26], [27].

The stretching vibrations of the C-N and C-H groups were linked to the absorption peaks at 1552 and 1311 cm^{-1}, respectively, and were identified as amides II and III [2], [11], [25]. In addition, three absorption peaks at 2920, 3104, and 3450 cm^{-1}, associated with the stretching of C=O, N–H, and O-H groups, respectively, were also observed in the CG2–5% extracted chitin and commercial shrimp chitin [1], [4]. However, the absorption at 3450 cm^{-1} was not clearly distinguishable owing to overlapping protein peaks in CE-extracted chitin [2], [19]. This implies that the biopolymer's structure was preserved following the CG2–5% extraction procedure. The FTIR spectra for the CG2-extracted chitin appeared to closely resemble those of commercial chitin.

To better understand biopolymer crystallinity, the XRD patterns of commercial shrimp chitin and the extracted chitin samples were analyzed, as depicted in Figure 2. The results showed that all extracted chitin displayed six prominent peaks at approximately 9.3, 12.7, 19.5, 20.8, 23.4, and 26.3°. Specifically, both the extracted and commercial chitin displayed two major diffraction peaks at 9.3 and 19.5°, along with four minor diffraction peaks at 12.9, 20.8, 23.4, and 26.3°. These peaks suggested that chitin is a stable structure of α-chitin [2], [13]. The peak positions and amplitudes align well with those documented for other chitin sources, such as Litopenaeus vannamei and *Penaeus*

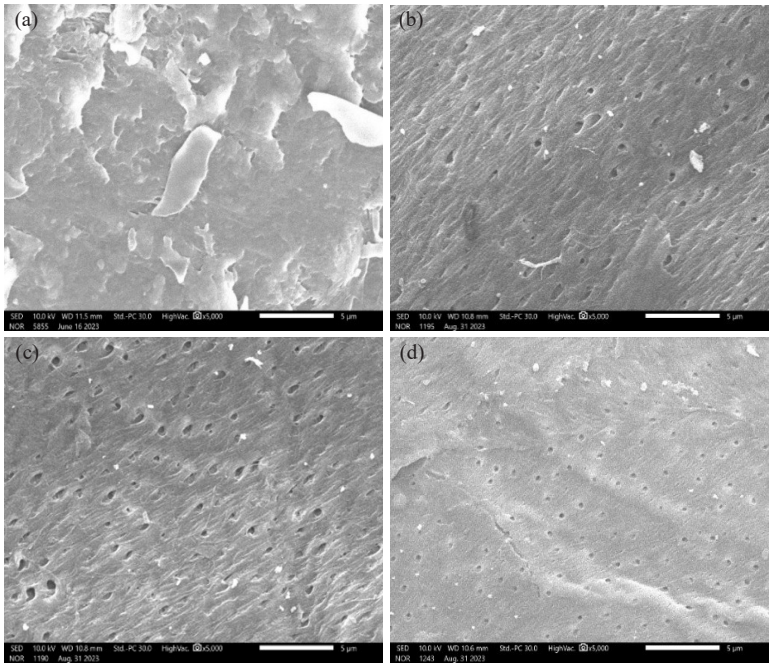

Figure 3: SEM images of (a) raw material, (b) CE-extracted chitin, (c) CG2–5% extracted chitin, and (d) commercial shrimp chitin with the magnifications are 5,000x.

monodon [2]. Furthermore, the Crystallinity Index (CrI) of the chitin samples, determined using the Segal method, revealed that chitin extracted via the CG2–5% process had higher CrI values (81.34%) than that extracted via CE (73.59%). Improved crystallinity suggests that mineral salts and proteins are removed from the shrimp shells [2], [27]. Simultaneously, the findings implied that the relatively reduced CrI of chitin, acquired via CE, could be due to the disruption of both intramolecular and intermolecular hydrogen bonds, along with the formation of amorphous chitin [27].

The morphologies of the shell and chitin samples after extraction from giant tiger prawns were compared using SEM. The untreated shrimp shell, as illustrated in Figure 3(a), exhibited a rough, non-porous surface because of the existence of mineral salts and proteins. In contrast, the chitin samples that were extracted possessed smooth surfaces with pores [Figures 3(b)–(d)]. Notably, the commercial chitin and CG2–5% chitin presented larger pore sizes on their surfaces compared to CE chitin [Figure 3(c) and (d)]. This can be attributed to the hydrolysis of chitin structures into small molecular weights by strong chemicals [4], [28]. The changes in the molecular weights (Mw) and the degree of deacetylation (DA) of the chitin extracted at different citric acid concentrations were investigated and compared with the data of chitin extracted by the conventional method and commercial chitin. In Table 7, it can be seen that the Mw and DA of chitin extracted using CG2 with 5% citric acid were 3.75×10^5 Da and 91.65%, respectively. These figures were slightly higher than those of chitin extracted through the traditional acid/alkali method (3.14×10^5 Da and 88.35%). This result suggests that CG2 with 5% citric acid inflicts less damage on the acetyl groups of chitin than strong acid and alkali, a finding similarly described with SEM results (Figure 3). According to Zhao *et al.*, the process of acid/alkali extraction weakens the intermolecular hydrogen bond of chitin, making the NaOH solution more effective in contacting and removing the acetyl groups of the chitin, resulting in a decrease in the DA value [4]. Simultaneously, there was no significant difference between the chitin extracted by CG2 with 5% citric acid and commercial chitin (4.12×10^5 Da and 91.91%). This indicates that CG2 with 5% citric acid effectively eliminated proteins and mineral salts from shrimp shells, resulting in high-purity chitin with minimal degradation.

Table 7: Crystallinity index (CrI) of previous and present studies

Sources	Crystalline Index (CrI, %)	Molecular Weight (Mw, Da)	Degree of Deacetylation (DA, %)	References
Raw material	$66.90^{c} \pm 1.13$	-	-	Present study
CE	$73.59^{b} \pm 1.78$	3.24×10^5	88.35	
CG2–5% chitin	$81.34^{a} \pm 1.21$	3.75×10^5	91.65	
Commercial shrimp chitin	$82.18^{a} \pm 1.38$	4.12×10^5	91.91	
Crab	67.8	-	-	[2]
Shrimp	88; 79.4–87.4	-	-	[21], [26], [29]

Note: Values with different lowercase letters in each column indicate a significant difference at $p < 0.05$.

4 Conclusions

A deep eutectic solvent (DES) was prepared from choline chloride (ChCl) and glycerine (Gl), and then it was combined with citric acid to extract chitin from giant tiger prawn shrimp. The purity and physicochemical properties of the chitin were measured and compared to those obtained from the conventional method and commercial shrimp chitin. The results revealed a positive correlation between the purity of the chitin and the concentrations of citric acid (0–5.0%) (CG2–5%). The purity of the CG2–5% chitin reached a peak of 99.22% at 80 °C, 4 h, and a 1:2 solvent to solid ratio. The purity of the chitin obtained was slightly higher than that of the conventionally extracted chitin. The molecular weight and degree of deacetylation of the chitin derived using CG2-5% were 3.75×10^5 Da and 91.65%, correspondingly, which were similar to commercial chitin (4.12×10^5 Da and 91.91%) and marginally higher than traditionally extracted chitin (3.24×10^5 Da and 88.35%). Analysis of the chemical composition, FTIR, XRD, and SEM confirmed that the chitin extracted with CG2–5% closely resembled commercial chitin, with no significant degradation occurring during the extraction process. Thus, the developed extraction method, which uses the eco-friendly CG2 (ChCl-Gl) and a small amount of citric acid (5%), demonstrated significant potential for the green and sustainable production of chitin.

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Author Contributions

T.C.H.: contributed to the conceptualization, investigation, review, and editing processes; N.T.D.: participated in the investigation and methodology; Q.L.A.T., H.L.T.N., N.D.T.T., N.T.T., and D.N.Q.: were all involved in the research design and data analysis; L.T.H.A.: wrote the original draft; P.V.M.: was responsible for the conceptualization, data curation, and the review and editing of the writing. Every author has read and given their approval to the final, published version of the manuscript.

Conflicts of Interest

The authors declare no conflict of interest.

References

[1] P. Ramasamy, N. Subhapradha, V. Shanmugam, and A. Shanmugam, "Extraction, characterization and antioxidant property of chitosan from cuttlebone *Sepia kobiensis* (Hoyle 1885)," *International Journal of Biological Macromolecules*, vol. 64, pp. 202–212, Mar. 2014, doi: 10.1016/j.ijbiomac.2013.12.008.

[2] K. Mohan, T. Muralisankar, R. Jayakumar, and C. Rajeevgandhi, "A study on structural comparisons of α-chitin extracted from marine crustacean shell waste," *Carbohydrate Polymer Technologies and Applications*, vol. 2, Dec. 2021, Art. no. 100037, doi: 10.1016/j.carpta.2021.100037.

[3] Z. Li, C. Liu, S. Hong, H. Lian, C. Mei, J. Lee, Q. Wu, M. A. Hubbe, and M. C. Li, "Recent advances in extraction and processing of chitin using deep eutectic solvents," *Chemical Engineering Journal*, vol. 446, no. 2, 2022,

Art. no. 136953, doi: 10.1016/j.cej.2022.136953.
[4] D. Zhao, W. C. Huang, N. Guo, S. Zhang, C. Xue, and X. Mao, "Two-step separation of chitin from shrimp shells using citric acid and deep eutectic solvents with the assistance of microwave," *Polymers (Basel)*, vol. 11, no. 3, 2019, Art. no. 409, doi: 10.3390/polym11030409.
[5] F. A. A. Sagheer, M. A. Al-Sughayer, S. Muslim, and M. Z. Elsabee, "Extraction and characterization of chitin and chitosan from marine sources in Arabian Gulf," *Carbohydrate Polymers*, vol. 77, no. 2, pp. 410–419, Jun. 2009. doi: 10.1016/j.carbpol.2009.01.032.
[6] M. Maddaloni, I. Vassalini, and I. Alessandri, "Green routes for the development of chitin/chitosan sustainable hydrogels," *Sustainable Chemistry*, vol. 1, no. 3, pp. 325–344, 2020, doi: 10.3390/suschem1030022.
[7] B. K. Park and M. M. Kim, "Applications of chitin and its derivatives in biological medicine," *International Journal of Molecular Sciences*, vol. 11, no. 12, pp. 5152–5164, 2010, doi: 10.3390/ijms11125152.
[8] N. M. Alves and J. F. Mano, "Chitosan derivatives obtained by chemical modifications for biomedical and environmental applications," *International Journal of Biological Macromolecules*, vol. 43, no. 5, pp. 401–414, 2008, doi: 10.1016/j.ijbiomac.2008.09.007.
[9] F. Khoushab and M. Yamabhai, "Chitin research revisited," *Marine Drugs*, vol. 8, no. 7, pp. 1988–2012, 2010, doi: 10.3390/md8071988.
[10] G. A. F. Roberts, *Chitin Chemistry*. UK: The Macmillan Press LTD, pp. 20–33, 1992, doi: 10.1007/978-1-349-11545-7.
[11] S. W. Kim, *Chitin, Chitosan, Oligosacharides, and their Derivaties: Biological Activities and Applications*. Florida: CRC Press, pp. 37–42, 2010.
[12] D. Jose, N. Kitiborwornkul, M. Sriariyanun, and K. Keerthi, "A review on chemical treatment methods of lignocellulosic biomass: Recent advances and progress," *Applied Science and Engineering Progess*, vol. 15, no. 4, 2022, Art. no. 6210, doi: 10.14416/j.asep.2022.08.001.
[13] J. Zhang, W. R. Xu, and Y. C. Zhang, "Facile production of chitin from shrimp shells using a deep eutectic solvent and acetic acid," *Royal Society of Chemistry Advances*, vol. 12, pp. 22631–22638, 2022, doi: 10.1039/d2ra03417d.
[14] S. Areeya, E. J. Panakkal, M. Sriariyanun, T. Kangsadam, A. Tawai, S. Amornraksa, U. W. Hartley, and P. Yasurin, "A review on chemical pretreatment of lignocellulosic biomass for the production of bioproducts: Machanism, challenges, and applications," *Applied Science and Engineering Progess*, vol. 16, no. 3, 2023, Art. no. 6767, doi: 10.14416/j.asep.2023.02.008.
[15] P. S. Saravana, T. C. Ho, S.-J. Chae, Y.-J. Cho, J.-S. Park, H.-J Lee, and B.-S. Chun, "Deep eutectic solvent-based extraction and fabrication of chitin films from crustacean waste," *Carbohydrate Polymers*, vol. 195, pp. 622–630, 2018, doi: 10.1016/j.carbpol.2018.05.018.
[16] J. Xie, W. Xie, J. Yu, R. Xin, Z. Shi, L. Song, and X. Yang, "Extraction of chitin from shrimp shell by Successive two-step fermentation of *Exiguobacterium profundum* and *Lactobacillus acidophilus*," *Frontiers in Microbiology*, vol. 12, 2021, Art. no. 677126, doi: 10.3389/fmicb.2021.677126.
[17] K. Morgan, C. Conway, S. Faherty, and C. Quigley, "A comparative analysis of conventional and deep eutectic solvent (Des)-mediated strategies for the extraction of chitin from marine crustacean shells," *Molecules*, vol. 26, no. 24, 2021, Art. no. 7603, doi: 10.3390/molecules26247603.
[18] Y. Wang, Y. Yang, R. Wang, Y. Zhu, P. Yang, Z. Lin, Z. Wang, and W. Cong, "Effectively inhibiting the degradation of chitin during extraction from crustacean waste via a novel deep eutectic solvent aqueous solution," *Process Biochemistry*, vol. 121, pp. 142–151, 2022, doi: 10.1016/j.procbio.2022.06.029.
[19] X. Sun, Q. Wei, Y. Yang, Z. Xiao, and X. Ren, "In-depth study on the extraction and mechanism of high-purity chitin based on NADESs method," *Journal of Enviromental Chemical Engineering*, vol. 10, no. 1, 2022, Art. no. 106859, doi: 10.1016/j.jece.2021.106859.
[20] Y. Qin, X. Lu, N. Sun, and R. D. Rogers, "Dissolution or extraction of crustacean shells using ionic liquids to obtain high molecular weight purified chitin and direct production of chitin films and fibers," *Green Chemistry*, vol. 12, no. 6, pp. 968–997, 2010, doi: 10.1039/c003583a.

[21] N. J. Kruger, "The Bradford Method for protein quantitation" in *The Protein Protocols Handbook*, W. J. Walker, Eds. New Jersey: Human Press, pp. 11–15, 2002.

[22] M. Feng, X. Lu, J. Zhang, Y. Li, C. Shi, L. Lu, and S. Zhang, "Direct conversion of shrimp shells to: O -acylated chitin with antibacterial and anti-tumor effects by natural deep eutectic solvents," *Green Chemistry*, vol. 21, no. 1, pp. 87–98, 2019, doi: 10.1039/c8gc02506a.

[23] W. Wang, S. Bo, S. Li, and W. Qin, "Determination of the Mark-Houwink equation for chitosans with different degrees of deacetylation," *International Journal of Biological Macromolecules*, vol. 13, pp. 281–285, 1991.

[24] D. Skarpalezos and A. Detsi, "Deep eutectic solvents as extraction media for valuable flavonoids from natural sources," *Applied Science*, vol. 9, no. 9, 2019, Art. no. 4169, doi: 10.3390/app9194169.

[25] B. Bradić, U. Novak, and B. Likozar, "Crustacean shell bio-refining to chitin by natural deep eutectic solvents," *Green Processing and Synthesis*, vol. 9, no. 1, pp. 13–25, 2020, doi: 10.1515/gps-2020-0002.

[26] M. Bisht, I. P. E. Macário, M. C. Neves, J. L. Pereira, S. Pandey, R. D. Rogers, J. A. P. Coutinho, and S. P. M. Ventura, "Enhanced dissolution of chitin using acidic deep eutectic solvents: A sustainable and simple approach to extract chitin from crayfish shell wastes as alternative feedstocks," *ACS Sustainable Chemistry and Engineering*, vol. 9, no. 48, pp. 16073–16081, 2021, doi: 10.1021/acssuschemeng.1c04255.

[27] M.-T. Yen and J.-L. Mau, "Physico-chemical characterization of fungal chitosan from shiitake stipes," *LWT - Food Science and Technology*, vol. 40, no. 3, pp. 472–479, 2007, doi: 10.1016/j.lwt.2006.01.002.

[28] M.-K. Jang, B.-G. Kong, Y.-I. Jeong, C. H. Lee, and J.-W. Nah, "Physicochemical characterization of α-chitin, β-chitin, and γ-chitin separated from natural resources," *Journal of Polymer Science Part A: Polymer Chemistry*, vol. 42, no. 14, pp. 3423–3432, Jul. 2004, doi: 10.1002/pola.20176.

[29] O. P. Gbenebor, S. O. Adeosun, G. I. Lawal, S. Jun, and S. A. Olaleye, "Acetylation, crystalline and morphological properties of structural polysaccharide from shrimp exoskeleton," *Engineering Science and Technology, an International Journal*, vol. 20, no. 3, pp. 1155–1165, 2017, doi: 10.1016/j.jestch.2017.05.002.