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Crustacean shell bio-refining to chitin by natural deep eutectic solvents

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Abstract: This study provides a complete evaluation of a sustainable zero-waste process for the recovery of added value biomaterials from the abundant shrimp shell biomass waste using natural deep eutectic solvents (NADES). The process parameters for the fractionation of α -chitin, minerals and protein was followed using on-line measurements. Furthermore, the quantitative analysis of isolated chitin, minerals and solvent waste streams were examined. The dominant fractionation mechanisms are explained through the analysis of the liquid and solid fractions. Four of the most promising, and commercially available, NADES consisting on mixtures of Choline Chloride-Lactic Acid (CCLA), Choline Chloride-Malonic Acid (CCMA), Choline Chloride-Urea (CCUR) and Choline Chloride-Citric Acid (CCCA), were tested. The highest chitin extraction yield obtained was < 90% using CCLA, leading to purity higher than 98%. Moreover, it is possible to recycle this particular NADES several times, while having no loss in the shrimp shell fractionation capability.

Keywords: chitin biopolymer isolation and characterization; natural deep eutectic solvents; green processing; zero-waste biorefinery process

1 Introduction

The worlds' production and consumption of seafood are increasing, with shrimp shells accounting for the current major seafood production waste [1]. The generated waste during the industrial shrimps processing reaches 50-70% of the raw shrimp weight. Therefore, food industry is motivated to use the accumulated crustacean biomass waste, rather than just discarding it back into

the seas and oceans [2]. Shrimp shells consist of three main components, namely proteins (30-40%), mineral salts (30-50%) and chitin (20-30%), while also having small amounts of lipids and pigments, all with a market value upon their isolation [3,4]. Chitin is considered the second most abundant organic resource on earth, next to cellulose, with an annually estimated worldwide natural production rate of approximately 10^{11} tons [3]. It is the only natural nitrogenous polysaccharide, i.e., a copolymer of N-acetyl-D-glucosamine and D-glucosamine units linked by a β -(1-4) glycosidic bond, and it is structurally similar to cellulose, having acetamido groups at the C-2 positions instead of the hydroxyl group [5]. Chitin can be converted to glucosamine, chitosan, oligosaccharides and N-acetylglucosamine [6]. Hence, chitin and its derivatives have become of great interest not only as an under-utilized resource but also as new functional biomaterial of high potential in several fields, such as biomedicine, cosmetics, food and environmental protection [7-10]. The industrial chitin isolation process encompasses two distinct chemical steps, namely demineralization and deproteinization, which are performed under high temperature and may also include the use of strong acid and alkali. The resulted waste from the conventional chemical extraction process is extremely hazardous to the environment, costly and consumes high concentrations of mineral acids with a large amount of freshwater. Additionally, the washing steps give rise to enormous volumes of polluted effluents that are technically challenging and expensive to recycle [11]. As alternatives, biological methods have been proposed, for instance, enzymatic reactions and microbial fermentation; however, these have not yet been applied in larger scale due to the longer fermentation cycles and expensive enzymes required [12]. Recently, our group proposed a unique solvent-less highly efficient option using the plasma-based methodology to pre-treat the crustacean shell waste, while allowing an intensified protein removal. This renewable electricity-based separation can serve as a scalable green alternative to the conventional chemical deproteinization step applying unrecyclable mineral bases [13]. Another alternative is the selective extraction of chitin with deep eutectic solvents (DES), since these have already shown great potential as dissolution media for some hardly soluble biopolymers, including

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cellulose, lignin and starch [14-16]. DES is composed of two or three cheap and safe components that self-associate through hydrogen bond interactions, forming an eutectic mixture with a melting point lower than that of each component [17]. Thus, being considered greener solvents due to their benign and environmentally sustainable preparation involving natural compounds. Additionally, DES properties, such as freezing point, conductivity, density, and viscosity, can be tuned according to its final application by the proper selection of the hydrogen bond donor (HBD) and hydrogen bond acceptor (HBA). Many plant abundant primary metabolites changed their state from solid to liquid when they were mixed in the proper ratio. This finding creates a new type of DES called natural deep eutectic solvents (NADES). To synthesize NADES, only natural based sources need to be used [18]. The use of DES in the extraction and/or dissolution of chitin have been proposed by several authors [18-24]. In one hand, authors of [17] reported the enhanced chitin dissolution by DES using conventional heating, heating under microwave irradiation and heating assisted by ultrasonication under an inert atmosphere. On the other hand, authors of [19] and [22] proposed the direct extraction of chitin from lobster and shrimp (*Marsupenaeus japonicas*) shells using DES composed from choline chloride as a hydrogen bond acceptor and urea, thiourea, glycerol and malonic acid as hydrogen bond donors at high temperatures except for the last one where 50°C was used, yet there was no recycling of the solvents. This was later improved in [20], where the authors performed a direct conversion of shrimp shells to O-acylated chitin using natural deep eutectic solvents (NADES) as a reaction medium, while also being able to remove the minerals and proteins simultaneously.

Finally, NADES were recycled and reused five times, though with some efficiency loss. Authors of [21] have also applied DES for the chitin extraction from shrimp shells, though herein authors proposed a two-step approach to fractionate the biomass into the different compounds, namely chitin, minerals and proteins. Minerals were obtained after a first citric acid treatment while the chitin and proteins fractionation was carried out with DES under microwave irradiation. DES reuse was also evaluated; however, their performance decreases after three cycles. In the current study, the zero-waste approach using NADES for the shrimp shell waste fractionation into chitin and minerals have been conducted, while having almost no loss in the NADES yield, upon proper optimization. Using advanced analytical together with the on-line probe the additional insides to the NADES-based shrimp shell fractionation mechanism was proposed and described

in the text. Four types of NADES consisting of choline chloride-lactic acid (CCLA); choline chloride-malonic acid (CCMA); choline chloride-citric acid (CCCA) and choline chloride-urea (CCUR) were tested in appropriate molar ratios. The shrimp shell fractionation process was optimized using a dynamic measurement of the particles with the on-line Focused Beam Reflectance probe (FBRM). After the biomass dissolution, chitin was precipitated and its fraction isolated from minerals. Additionally, the dominant mechanisms for the fractionation are explained based on the systematic analysis of the liquid and solid fractions. Chitin and mineral purity was further characterized by several advanced analytical tools while additional analysis was performed for liquid fractions after chitin precipitation. Lastly, the recyclability of all the used NADES and process water was studied and their influence on the chitin yield and purity tested.

2 Materials and methods

2.1 Material

Lactic acid 85% FCC, malonic acid, citric acid, urea, N, N-dimethylacetamide (DMAc) and lithium chloride were purchased at Sigma Aldrich (Steinheim, Germany). Sodium hydroxide pellets, hydrochloric acid (32%), all of the analytical grade were supplied by Merck KGaA (Darmstadt, Germany). Shrimp shells waste from the deep-water shrimp (*Pandalus borealis*) obtained from a local factory in northern Norway in the form of powder and stored in a sealed container at -18°C until further use.

2.2 Preparation of NADES

Both the HBD: urea, lactic acid, citric acid, malonic acid and HBA: choline chloride were weighted in a flask at the defined molar proportion and mixed for at least 2 h at a temperature of 80°C until the liquid solution was obtained. Molar ratio 1:1 was used for NADES composed of (CCLA); choline chloride-malonic acid (CCMA); choline chloride-citric acid (CCCA), while molar ratio 1:2 was used in the case of choline chloride-urea (CCUR), respectively.

2.2.1 NADES viscosity determination

The intrinsic viscosity of all NADES was determined using rotational viscometer Fungilab S.A. (New York, USA).

A standard TL7 spindle with the rotation speed of 50 rpm, for 10 s at room temperature (24°C) was used.

2.3 NADES-based extraction and purification of chitin from shrimp shell powder

Chitin isolation from shrimp shells was carried out in a mixing reactor with a volume of 250 mL at 60°C, 70°C, 80°C and 90°C. 1 or 2 g of shrimp shells powder was dissolved in 50 g of NADES. Firstly, the distribution of powder shrimp particles was determined with in-line particle measurements using METTLER TOLEDO FBRM® G400 probe (Mettler Toledo, Columbus, Ohio, USA), which was inserted directly into NADES. The particle size distribution was measured immediately after the addition of the particles with iC FBRM™ Software

and the number of particles in a selected size range was acquired. The FBRM probe was immersed in a suspension at the 45° angle. Assessment of the particle size was performed by measuring the number of laser beam interruptions with constant circumferential speed, which gave the distribution of particle chord lengths [25,26]. At least three experiments were performed, being the average values calculated. For the temperature studies, 3 h and 6 h of dissolution time were used. After the shrimp biomass dissolution, 100 mL of water was added to the suspension and mixed for several minutes. The suspension was filtered through 0.45 µm filters, using vacuum filtration pump to obtain the solid and liquid fractions. The solid phase was then collected and dried in an oven at 105°C to constant weight and further characterized using XPS, XRD, SEM, EDS and FTIR. The integrated process is shown in Figure 1.

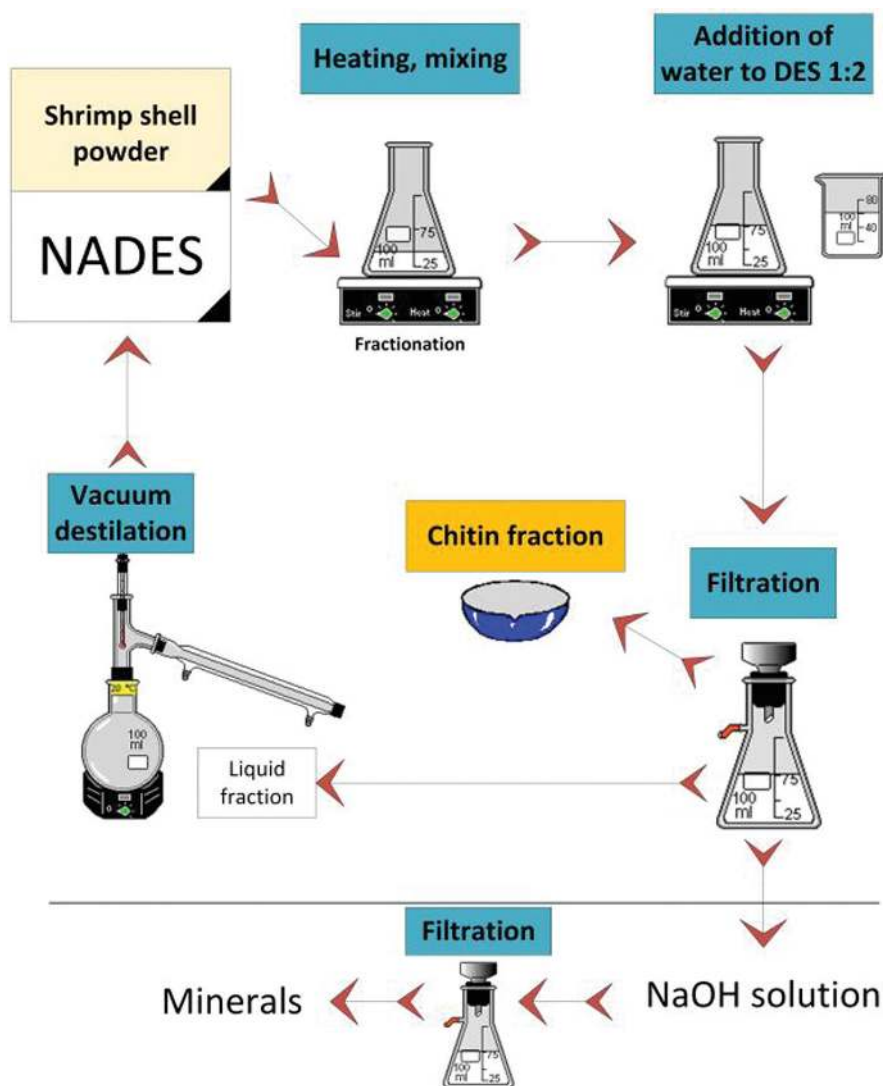


Figure 1: Sustainable process for chitin and minerals fractionation from shrimp shell by NADES.

2.4 Recycle of NADES and minerals separation

Recycled NADES was obtained from the liquid fraction after the filtration step. The obtained filtrate was subjected to vacuum distillation until the water was removed (40 mbar, 40°C, 6 h). The minerals separation was carried out by adding a NaOH solution at different concentrations, depending on the NADES under study. The volume ratio of NADES to NaOH was 2:1 and the used NaOH concentration for CCLA, CCMA and CCCA were 70 wt%, while for the CCUR it was 80 wt%, respectively. The precipitate was filtered and dried before being characterized using SEM and EDS.

2.5 Chitin determination

Chitin content was determined using the method described in [27]. 0.2-0.4 g of the dried sample was placed in a beaker with 50 mL of 1M HCl and heated for 1 h at 105°C. The sample was filtered through sintered glass crucible and washed with distilled water. The residue was placed back into a beaker with 100 mL of 5% NaOH solution and heated for 1 h at 105°C. Shrimp shells were filtered through sintered glass crucible and washed twice with distilled water and twice with 15 mL of acetone. Samples were dried in a crucible at 110°C to constant weight and incinerated the content in a furnace at 600°C for 6 h. The weight loss in the incineration step represents the chitin content in a sample.

2.6 Determination of the degree of deacetylation for isolated chitin samples

The degree of deacetylation (DDA) for the isolated chitin samples were determined using the ATR-FTIR method proposed in [28]. ATR-FTIR spectroscopy of isolated chitin samples was performed with a Spectrum two (Perkin Elmer, Manchester, UK) using LiTaO₃ MIR detector over the frequency range 400-4000 cm⁻¹ at the resolution of 4 cm⁻¹. DDA was calculated using Eq. 1, where the dominated peak at 1655 cm⁻¹ presented the overlapped bonds of NH and C=O, and bands at 3450 cm⁻¹ presented NH-C=O stretching vibration. Both chosen peaks are dominated in chitin samples. The values obtained for each sample corresponding to the average value of five spectra.

$$\text{DDA}\% = \left(A_{1655} / A_{3450} \right) \times 115\% \quad (1)$$

2.7 Molecular weight determination of chitin samples

The average molecular weights (MW) of chitin were calculated from measured intrinsic viscosities using the Mark-Houwink relationship (Eq. 2):

$$\eta = K M_w^a \quad (2)$$

where η is the intrinsic viscosity, M_w the viscosity average molecular weight and K and a are constants for the given solute-solvent system and temperature. Determinations of chitin molecular weight were determined as described in [29]. Chitin samples were solubilized in 0.25 mg/mL in N,N-dimethylacetamide (DMAc) solution containing 5 wt% LiCl. The values for the constants a and K were 0.95 and 7.5×10^{-5} (dL g⁻¹), respectively.

2.8 X-ray photoelectron spectroscopy (XPS)

The X-ray photoelectron spectroscopy (XPS or ESCA) analyses were carried out using the PHI-TFA XPS spectrometer produced by Physical Electronics Inc. Sample powders were deposited on conducting carbon adhesion tape. The analyzed area was 0.4 mm in diameter and the analyzed depth was about 3-5 nm. This high surface sensitivity is a general characteristic of the XPS method. Sample surfaces were excited by X-ray radiation from monochromatic Al source at a photon energy of 1486.6 eV. The high-energy resolution spectra were acquired with energy analyzer operating at a resolution of about 0.6 eV and pass energy of 29 eV. During data processing, the spectra from the surface were aligned by setting the C 1s peak at 285.0 eV, characteristic for C-C bonds. The accuracy of binding energies was about ± 0.3 eV. Quantification of surface composition was performed from XPS peak intensities taking into account relative sensitivity factors provided by the instrument manufacturer. Two-three different XPS measurements were performed on each sample and average composition was calculated.

2.9 Powder X-ray diffraction (XRD)

Powder X-ray diffraction (XRD) studies were conducted using the PANalytical X'Pert Pro instrument. Scanning from 5 to 90° was carried out using the CuK α radiation source with a wavelength of 0.15406 nm.

2.10 Scanning electron microscopy (SEM)/Energy-dispersive X-ray spectroscopy (EDS)

Chitin samples isolated with different eutectic solvents were structurally characterized by using scanning electron microscopy (SEM) (SUPRA 35 VP, Carl Zeiss, Jena, Germany) operating at 1 kV. Electron microscopy was performed at high magnification, which generated high-resolution images and was used to precisely measure very small changes in the sample features.

2.11 Wavelength-dispersive X-ray fluorescence spectroscopy (WD-XRF)

Rigaku Supermini200 equipped with three crystals RX25, PET, LiF200 and has a fixed 30 mm collimator adjusted at 0.30° (Rigaku, Neu-Isenburg, Germany). As a primary beam filter zirconium 0.200 mm was used. For all the measurements, the conditions were 50 kV and 4 mA. The detector limits for the elements are listed in Table S2 in Supplementary material.

3 Results and discussion

3.1 Fractionation of shrimp shell biomass into valuable components using NADES solution

Chitin is predominantly located in the inner layers of the shell exoskeleton alongside proteins, assisting in the shell sclerotization. The exoskeleton middle layer is composed of chitin with minerals, while the upper layer is made of calcium carbonate and proteins [30]. For successful chitin isolation, exfoliation of the outer and middle layers is required. Therefore, the studied NADES need to be able to play three roles: demineralization, deproteinization, and chitin dissolution. Demineralization, particularly for the

calcium carbonate removal, requires acidic conditions, leading to the common use of an organic acid as the HBD. As for the deproteinization, high temperatures are used to denature proteins while using acidic or alkaline conditions to hydrolyze them into aminoacids [31]. In this sense, the employed NADES in this study are based on citric, malonic and lactic acids as the HBD and choline chloride as the HBA, while being compared with a more conventional and alkaline approach, namely an urea-based NADES. When applied towards the chitin isolation, NADES mechanism has been explained as the agent breaking the strong hydrogen bonds in the chitin molecule through the reaction between the amino group of chitin and the H⁺ from the NADES components. Chitin dissolution in NADES was shown to increase the system viscosity due to the intense hydrogen bonds being formed between choline chloride and the biopolymer molecules, thus making its structure and the mobility of the NADES components more rigid [17,32]. To overcome this issue and reduce the mixture viscosity, the system temperature was increased. Nonetheless, this could also be accomplished by adding small amounts of water to the system. The studied molar ratios of NADES forming components and the final NADES viscosity obtained are presented in Table 1.

NADES viscosity ranges from 450 to 1250 mPas of CCLA and CCCA, respectively. The CCLA lowest viscosity value was a result of the addition of an 85% aqueous solution of lactic acid to choline chloride in order to form the NADES.

Regarding the dissolution kinetics of the shrimp shell powder, a total particle count measure was performed using an FBRM probe. In all the studied systems, the FBRM analytical technique was used to monitor the total number of particles in the chord size range from 1 to 1000 μm at 60°C or 80°C, depending on the NADES employed. As CCUR and CCCA display a considerably higher viscosity than the remaining systems, these were studied at a higher temperature to overcome this issue. The normalized particle counts for CCLA-60, CCMA-60, CCUR-80 and CCCA-80 are shown in Figure 2.

Table 1: Studied NADES with their abbreviations as well as the molar ratio between HBD and HBA and viscosity at 24°C.

Hydrogen bond acceptor (HBA)	Hydrogen bond donor (HBD)	Abbreviations of NADES	Molar ratio	Viscosity (mPas)
Choline chloride	Lactic acid (85%)	CCLA	1:1	450
Choline chloride	Malonic acid	CCMA	1:1	520
Choline chloride	Urea	CCUR	1:2	980
Choline chloride	Citric Acid	CCCA	1:1	1250

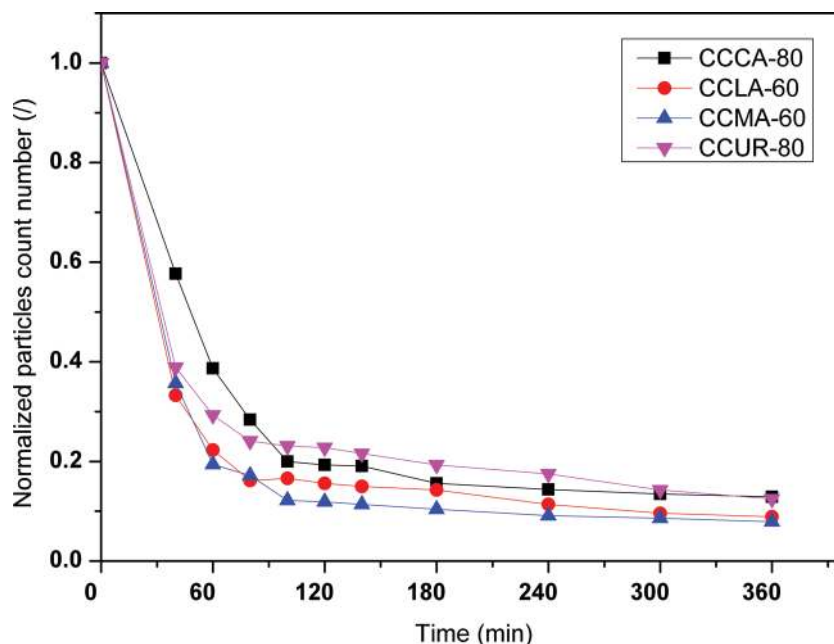


Figure 2: Shrimp shell powder dissolution dynamics in NADES, with the real-time measurement of total particle number (normalized) in the range from 1 to 1000 μm . Temperatures for different NADES were CCCA (80°C), CCLA (60°C), CCMA (60°C) and CCUR (80°C), 100 min^{-1} , 50 mL of NADES and 1 g of shrimp shell powder.

A fast decrease in the number of particles is observed within the first 2 h for all NADES. This confirms that the shrimp shell particles have been mostly dissolved or degraded, as later discussed in more detail. Yet, there is still ~10% and ~17% of the initial particle count detected for the case of CCMA-60 and CCLA-60, and CCUR-80 and CCCA-80, respectively. One of the factors that could be contributing to this might be related to the experimental set-up since it was noticed that with the more viscous liquids, there were more small-sized air bubbles entrapped in the liquid. By comparing the results for both applied temperatures, there was no significant difference in the final particles number after 6 h.

Chitin dissolution has been proven to be a result of the hydrogen bonds break within the biopolymer, being these replaced by new hydrogen bonds between chitin and choline chloride as well as by the reaction between the acetamido group in chitin and the free H^+ ions of choline chloride [33]. The preliminary research by [17] showed that NADES could effectively dissolve chitin, reaching up to 9 wt% of chitin dissolution with a choline chloride-thiourea-based DES. In this sense, the first step in the chitin dissolution should be the removal of the proteins, tightly bound to the chitin fibrils. Here this was accomplished by acid or alkaline hydrolysis at elevated temperature. The second step to reach chitin is the mineral removal, where most CaCO_3 reacts with the NADES acidic component, forming calcium salts, water and CO_2 .

In summary, once the shrimp powder has been dissolved in NADES, its fractionation into the different compounds can be preceded. This fractionation was carried out using two different solid (shrimp shell powder) to NADES ratio, namely 1:25 and 1:50, which were chosen based on the NADES capability to dissolve chitin and the solution viscosity. Highly viscous solutions make the fractionation much harder due to the slower diffusion of NADES into shrimp shells. The first fraction corresponds to the chitin isolation from initial suspension (Figure 1), which was accomplished by adding water to the solution. This results in the disruption of NADES structure and leads to the precipitation of water-insoluble fractions. The obtained yield for all NADES at both ratios and at two temperatures is shown in Figure 3a. From this figure, it is clear that the chitin fraction can reach up to 20 wt% from the maximum 23 wt% chitin present in the dried shrimp shell powder used. This represents a recovery yield of more than 85% of chitin from the initial shrimp shell biomass using CCLA-70. Furthermore, it can be observed that by increasing the shrimp powder mass in NADES, the chitin yields are reduced for most solvents and at both temperatures under study. The higher amount of particles could lead to saturation of the solvents fractionation capabilities and further limiting chitin mass transfer, due to the limited HBD capabilities of the NADES.

Regarding the temperature influence, it has been previously explained why 60°C and 80°C were used,

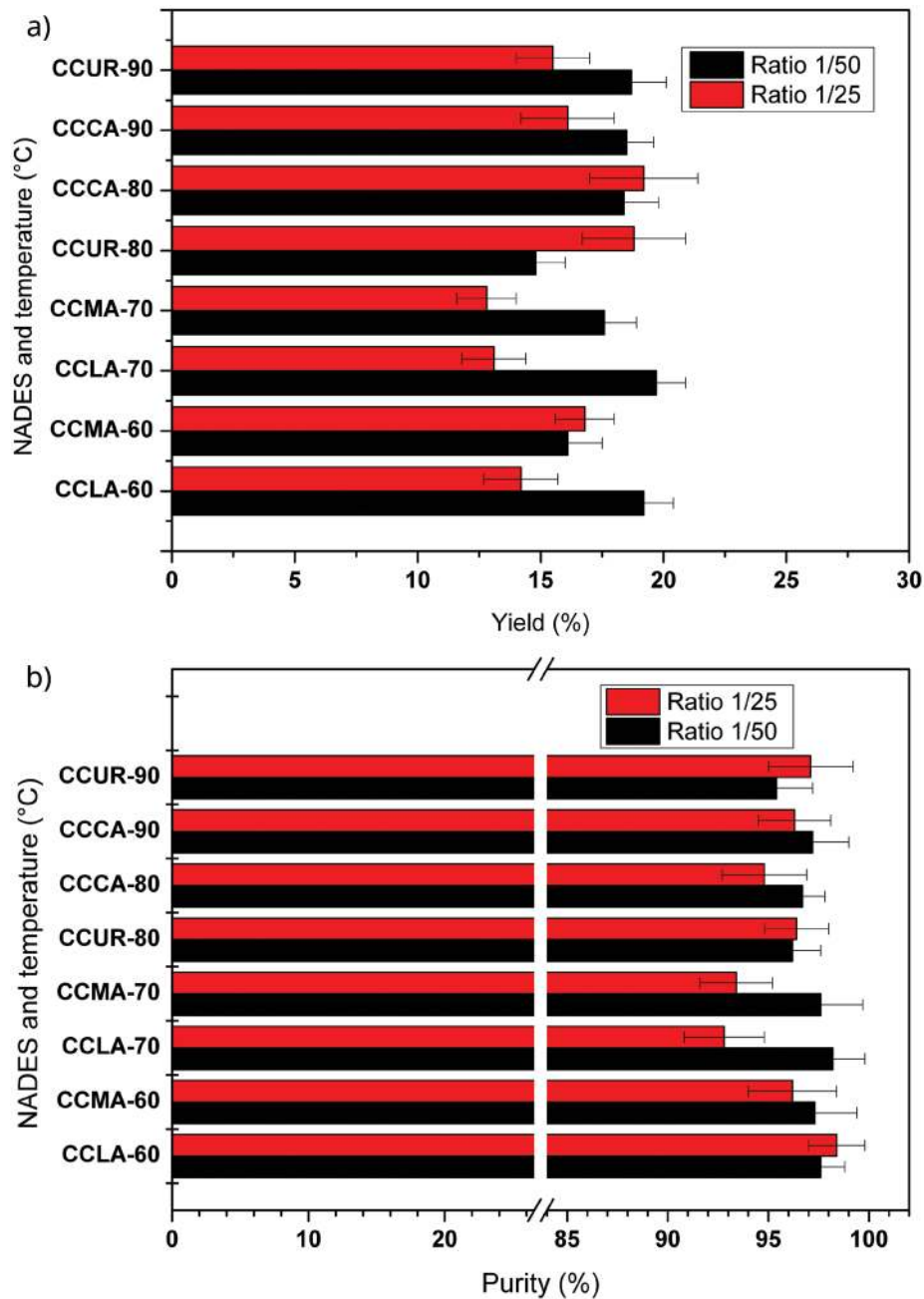


Figure 3: Chitin yield (a) and purity (b) obtained using NADES at different temperatures and shrimp shell powder ratios. Fractionation time of 6 h at 100 min⁻¹.

i.e., different NADES viscosity; nevertheless, in this particular study, 70°C and 90°C were also analyzed in order to evaluate if the temperature increase would be beneficial for the fractionation, since NADES viscosity decreases with temperature. As a result, there should be a faster and more effective interaction through hydrogen bonding, while also allowing a faster penetration of NADES inside of shrimp shell matrix removing the outer layers of proteins and minerals. Figure 3a shows

that the temperature increase is accompanied by a yield decrease for most systems with 1/25 ratios, is the difference in yields more pronounced in the case of lower temperatures CCMA-70 and CCLA-70-based NADES. Yet, by increasing the temperature using the same NADES at 1/50 ratio, there was an increase of chitin yield. In addition to the yield, chitin purity is a crucial parameter, so it has also been determined and is presented in Figure 3b.

Table 2: Gravimetrically determined purity of the isolated chitin and measured crystallinity index, molecular weight and degree of deacetylation of the polymer chain.

NADES	Purity (%)	Crystallinity Index (/)	Molecular weight (kDa)	DDA (%)
CCLA-70	98 ± 1	91	125	11
CCMA-70	98 ± 1	81	86	9
CCCA-90	97 ± 1	76	84	7
CCUR-90	95 ± 1	43	75	8

For all the studied NADES, the purity of the isolated chitin was impressive with values higher than 92%, being predominantly higher at the highest solid to NADES ratio. Two exceptions were yet observed for the CCLA-60 and CCUR-80-based systems, where there was no significant difference. A more detailed analysis was carried out considering the chitin crystallinity index, molecular weight and degree of deacetylation at the highest fractionation temperature, being these results shown in Table 2. FTIR spectra of the samples presented in Table 2 can be seen in Supplementary material (Figure S1).

Results (Table 2) showed that chitin polymorphism and its crystallinity are affected by the preparation procedure resulting in the molecular weight decrease of obtained polymers. The resulted generation of amorphous polymer structure can be due to the cleavage of intra and intermolecular hydrogen bonds [19-22,33]. The highest crystallinity index was obtained with CCLA-70 as well the highest degree of deacetylation, indicating that the degree of crystallinity depends on the chitin degree of deacetylation. This has also been observed in [34] and may be attributed to the fact that chitin chains with a higher degree of deacetylation are more flexible and have fewer large acetyl side groups.

The diffraction peak of CaCO_3 disappeared, which indicates that CCMA DES could remove CaCO_3 , which is the same as the effect of acids in the existing methods [19]. However, weak characteristic peaks of chitin and strong peaks of CaCO_3 in the samples obtained by CCUR, were observed in [19], suggesting that chitin was isolated simultaneously with CaCO_3 , an inorganic component of lobster shells.

In contrast, the chitin obtained using CCUR-90 showed the lowest crystallinity index, which could be due to the combination of the high pH conditions and the higher temperature used. Additionally, SEM images for the isolated chitin samples with different NADES, which showed a relation with porous structure of isolated samples and molecular packing of chitin chains (Figure S2). Regarding the literature data, SEM images showed that chitin with higher

molecular mass has a large surface with loose microfibrils [19,21]. Even lower MW of isolated chitin from the shrimp shell 79 kDa using CCMA-80 was obtained by authors of [17], who compared the MW of the chitin extracted using conventional method 132 kDa and a commercial standard 286 kDa. Hong et al. [23] measured the MW of the chitin extracted from the lobster shell using DES and observed big influence of the temperature used for the extraction in all DES. The MW ranged from 312 kDa for the choline chloride malonic acid at 50°C and using cold water for the filtration to 91 kDa for the choline chloride malic acid DES at 100°C and hot water used for the filtration. The lowest MW in our study was obtained by the CCUR-90, even though this DES showed the best chitin solubility properties and as shown in [17], where the MW of the chitin after dissolution in DES was not decreased significantly.

Moreover, the powder XRD, TGA and FT-IR results of the regenerated material were identical to those of the unprocessed α -chitin. Therefore the explanation of the high decrease of the MW should be in the mechanism of the fractionation, namely the way the proteins and minerals have been separated from the chitin. CCUR gives the lowest purity of the chitin and much lowest CI compare to other NADES tested, which leads to a conclusion that the morphology of the chitin has been affected by this solvent during its isolation. This might be the reason for the lower MW, although hydrolysis of the chitin chain is not expected to be favored by this NADES. In the case of [17] for the dissolution, the high MW commercial chitin with high CI was used. The crystallinity decreases due to the dissolution of the sample by NADES, which leads to the cleavage of intra and intermolecular hydrogen bonds and the generation of some amorphous chitin. Moreover, since the CCUR is known to have the highest solubility for the chitin, consequently its effect on the CI can be expected leading to the more amorphous chitin. Additionally, authors of [19] obtained chitin by CCMA DES, which could be divided into two parts with different crystallinity (67.2% and 80.6%), which also had different thermal stability, leading to the conclusion that NADES effect on the MW needs to be investigated even further.

As before mentioned, chitin fractionation from the shrimp shell should be carried out by firstly choose the more selective NADES towards minerals and proteins removal and not only by considering chitin dissolution, as it has been shown in this study that the NADES with the highest capacity for the chitin solubility is not the best option for the overall fractionation. CCUR-based NADES has been proven to be one of the best in dissolving more than 6 wt% of chitin [17], yet here is the worst solvent. Considering the comparison of the different organic

acids-based NADES, CCLA-70 appears to be the most promising NADES. Herein, an 85 vol% aqueous solution of lactic acid was used, showing a positive effect on protecting the hydrogen bonds network in the α -chitin biopolymer. The presence of the water also improves the reaction of the acid with the minerals and the acidic hydrolysis of the proteins. The domination of the hydrophilic character with a high amount of N-amino-D-glucosamine unit in the chitin backbone can be determined by the degrees of deacetylation. For all NADES, the measured DDA of chitin is quite low, making it insoluble in water. Further characterization, presented in Table 3, was performed on the isolated chitin using CCLA-70, since it was the one with the highest obtained purity.

XRD measurements allowed us to determine the presence of the minerals left after chitin dissolution and their further isolation. In contrast, XPS elemental analysis allowed the study of the polymer surface. Both methods showed higher values of impurities compared to the gravimetric method for chitin determination. The

reason for a higher percentage of impurities in the case of the XRD is that it is an arbitrary method since some peaks could overlap, while for the XPS, only outer 5 nm of the surface was analyzed. Thus, it might not be the most precise method. Not to mention, chitin might still be wrapped with some proteins and minerals. Nevertheless, the combination both of methods can give us the impurities percentage, where it can be seen that 49 wt% of the sample surface are proteins (based on determined nitrogen on the surface), 28 wt% is CaCO_3 and 23 wt% are other elements, mostly Cl and P.

3.2 Separation of minerals from NADES solution

Before recycling NADES, the separation of the mineral fraction can be carried out by precipitation with NaOH. The obtained minerals yields using different NADES are shown in Figure 4.

Table 3: Total impurities present in isolated chitin sample and the impurity composition in chitin. Complete XPS elemental analysis is available in (Table S1).

Impurities in the sample	Gravimetric method (%)	XRD method (%)	XPS method (%)	CaCO_3 (%)	Proteins (%)	Other - Cl, P, Si (%)
CCLA-70	2*	6*	5*	28**	49**	23**

* Represents the total % of the impurities in the chitin sample

** % of the CaCO_3 , proteins and other elements in the impurities (calculated from XRD and XPS)

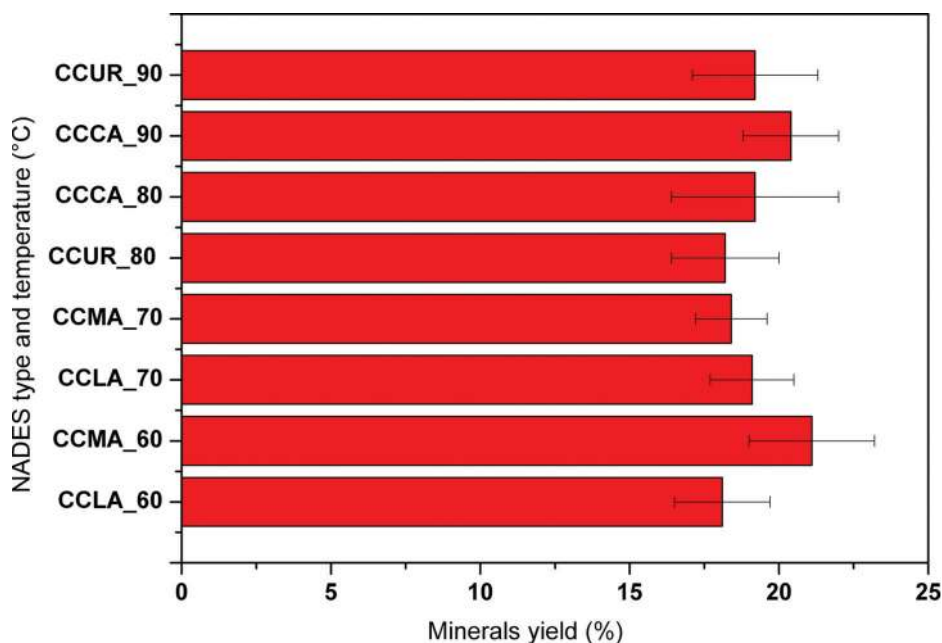


Figure 4: Mineral yield after precipitation with NaOH for the different NADES under study.

The yield values range from 16% to 22%, representing from 42% to 58% of the minerals initially present in raw shrimp shells. Mineral content was analysed using the SEM and EDS method, which gives the composition of the obtained fractions and is part of Supplementary material (Figures S3 and S4). Moreover, during the NADES fractionation and precipitation, minerals can undergo various changes in structure and form different mineral salts as well as degradation into CO₂ and water as side products, which in turn reduce the yield of isolated minerals (demineralization), especially in acidic NADES types. The analysis of the DES fraction showed a high amount of Ca in the DES, which results in the formation of the soluble fraction of the Ca, which are being retained in NADES after filtration. Additionally it has been already shown by others that the KOH can also interact with the DES forming component making new type of DES [15], NaOH can also be the case and could influence the more efficient deproteinization step therefore releasing the CaCO₃ (Figure S4). The EDS analysis of the isolated minerals showed a high percentage of the CaCO₃ and also some amount of the Na, P, Mg and S, which can be part of the salts. Since the NADES showed still a high amount of Ca in the system after the regeneration of NADES, with no addition of NaOH (Tables 4 and 5), concluding that the CaCO₃ is still in the structure matrix combined with proteins making them soluble in NADES. This shows that the purpose of the NaOH was to increase the deproteinization leading to the isolation of the CaCO₃, which is then precipitating, due to its insolubility in NADES.

Table 4: Composition of the NADES CCLA before and after shrimp dissolution, chitin isolation and NADES recycle.

	CH ₂ (%)	Cl (%)	Other (%)
Pure CCLA (NADES)	89.4	10.6	0.04
CCLA with dissolved shrimp shell	90.1	9.5	0.41
Recovered CCLA	94.4	5.3	0.34

Table 5: Percentage of Ca, Mg, S and other elements in the NADES mixtures excluding CH₂ and Cl.

	In total NADES mixture (%)	Ca (%)	Mg (%)	S (%)	P (%)	Other (%)
Pure CCLA (NADES)	0.04	13.4	55.8	0	1.7	29.1
CCLA with dissolved shrimp shells	0.41	76.3	7.9	0.3	10.7	5.1
Recovered CCLA	0.34	72.4	8.1	1.7	15.5	4

3.3 NADES recycle and reuse-liquid phase examination

For NADES recovery study, the minerals isolated using NaOH solution as was performed and explained in the previous section was not performed in this section. The main reason is reducing the number of steps and less chemical needed, making the fractionation process more friendly and effective on an industrial scale. In a single cycle, only a small amount of the mineral can be extracted compared to the solvent needed (ratio 1:50). Therefore is a reasonable solution that the NADES should be recycled several time, isolating only chitin (high purity and high yield) while the mineral fraction can be isolated after the decrease of the quality parameters of the obtained chitin. As can be seen in Figures 5a and 5b, NADES have been recycled and reused twice for chitin isolation at the ratio 1:50 due to the higher purity obtained in the preliminary studies (Figure 3b). Authors of [24] observed the same trend with the lower ratio of chitin in DES the demineralization and deproteinization efficiency was increased considerably, especially the latter.

These results indicate that, upon a carefully NADES selection, it is possible to select a system with almost no loss in the chitin isolation yield during its recycling (2%). It can be seen that the purity of isolated chitin decrease after first reusing of NADES (Figure 5b). Bearing in mind that in the re-use of NADES, the solvent reaction with chitin is limited, the viscosity of the solvent increases, and the saturation of NADES is expressed. It can be concluded that in the recycled NADES, the residual components from the shrimp shells fractions mostly affect the percentage of the impurities.

The dominant mechanism for fractionation could be explained by the systematical elemental study of the liquids in this sustainable circular process. Side products from the liquid phase precipitated chitin and process water were removed. Pure NADES solvent, dissolved shrimp shell and recovered NADES were further analyzed. The liquid composition is shown in Table 4 and consists of hydrocarbon source CH₂ and chloride (Cl), which are part of NADES, and other elements represent less than 0.5% of the liquid phase composition.

It can be seen that the presence of Cl is reduced during the fractionation and regeneration phase. In reused CCLA, % of Cl is lower by half, which could be the reason for the reduced efficiency of chitin isolation in recycling steps, affecting the capability of hydrogen bond formation

between chitin and reused NADES solution. Table 5 shows the relationships between other elements, which are not part of NADES.

As expected after the shrimp shells dissolution the P and Mg are detected, while the highest amount was elemental Ca. A similar trend is observed after chitin precipitation, and water evaporation, the wherein total ratio of the other element is slightly dropped from

0.41% to 0.34%, which is in concordance with the impurities detected in isolated chitin. The increase of the impurities in isolated chitin using recycled NADES can be correlated with the elements present in the pure NADES solution (0.04%) and dissolved shrimp shell components in NADES (0.41%) especially Ca, while the phosphorus (P) is increased. The detection limits for the WD-XRF instrument are presented in Table S2.

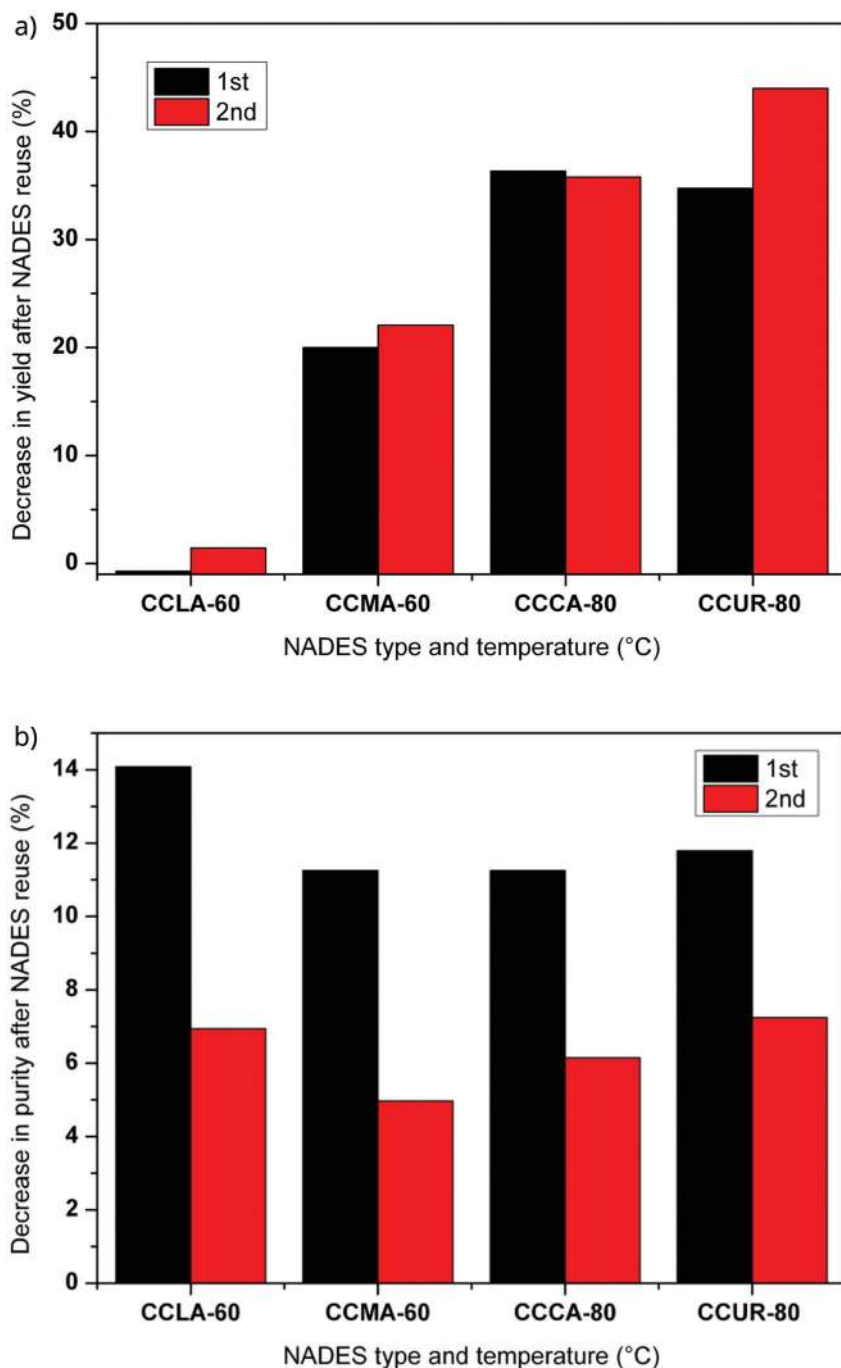


Figure 5: Influence of the NADES recycle and reuse on chitin (a) yield and (b) purity. The same conditions were used, as described in Figure 1.

4 Conclusions

The presented approach using NADES for fractionation of the shrimp shell waste can be considered a zero-waste process. Due to the unique multifunctional properties of the selected NADES, α -chitin can be separated from minerals and proteins in a single step. Other main components, minerals mostly in the form of CaCO_3 are removed by acid conditions, and proteins are being degraded at elevated temperatures using acid or base environment. NADES recycling and reusing is a significant feature for any environmental and economic aspect, which has been achieved by the proper selection of NADES, without loss in the chitin yield. In the future optimization regarding the isolation of other added value components from the shrimp shell waste but foremost making the chitin isolation a continuously operated process is foreseen.

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