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Low-Phytotoxic Deep Eutectic Systems as Alternative Extraction Media for the Recovery of Chitin from Brown Crab Shells

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Cite This: *ACS Omega* 2021, 6, 28729−28741

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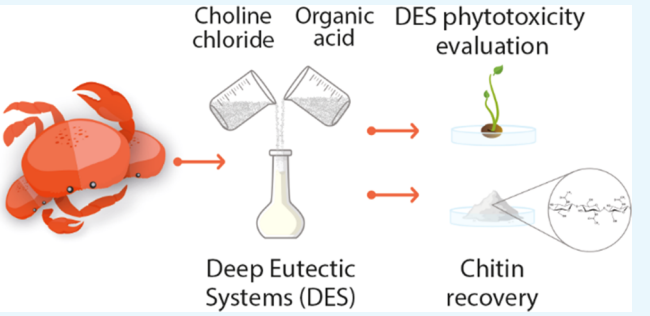
ABSTRACT: The versatility of chitin and its derivatives has allowed their utilization in a wide range of applications, from wastewater treatment to pharmaceutical or biomedical industries. However, even though the extraction method used industrially is extremely efficient, it involves the use of strong acids and bases and results in the disposal of large quantities of toxic effluents. Deep eutectic systems (DESs) have emerged as a promising new class of alternative solvents, including for chitin recovery. Yet, the assessment of their toxicity has often been neglected. Therefore, in this work, the phytotoxicity of choline chloride (ChCl)/organic acid-based DESs toward wheat seeds was evaluated by measuring different growth parameters and stress biomarkers. DESs were then explored for the efficient recovery of chitin contained in brown crab shell residues at varying conditions of temperature and processing time as well as with and without water addition. The obtained chitin was then characterized through different analytical techniques and compared to a standard as well as to chitin obtained by a conventional acid/alkaline hydrolysis. Results have shown that by applying a ChCl/lactic acid-based DES (which was the system that showed the least phytotoxic effects on wheat; EC50 ≥ 1.6 mg/mL) at 130 °C, it was possible to obtain pure chitin (up to 98%) with characteristics similar to those presented by commercial chitin or chitin recovered by conventional hydrolysis in a shorter time (more than 8-fold faster), thus suggesting that ChCl/organic acid-based DESs can truly represent a low-phytotoxic alternative extraction media for the recovery of chitin from the crab shell biomass.



INTRODUCTION

Chitin is a linear polysaccharide that plays a supportive and protective role in different living organisms.[1,2] In particular, chitin is one of the main constituents of crustaceans exoskeleton, a complex structure that provides the necessary mechanical strength to protect the soft body of crustaceans and comprises three layers: an inner layer formed by chitin and proteins, a middle layer composed of chitin and minerals, and an upper layer consisting of calcium carbonate and proteins.[3]

Due to their stabilizing and emulsifying properties, chitin and its derivatives have been used in the food industry to improve food safety, quality, and shelf-life.[2,4] Additionally, owing to properties such as low toxicity, biocompatibility, and biodegradability, chitin and chitosan have also been used as excipients and as biologically active agents in the cosmetic industry; or as biomaterials in the pharmaceutical and biomedical industries, including in bone and cartilage regeneration, wound healing and dressings, contact lenses, drug delivery systems, among others.[4−6]

The demand for this naturally occurring polymer and its derivatives has increased recently, triggering swift market growth, at a compound annual growth rate of 15.4% (from 2016 to 2021).[7]

In many countries, shellfish waste is already being used as a feedstock to produce chitin, chitosan, and glucosamine sulfate for a wide range of applications, especially in the biomedical field. This allows not only the maximization of the shellfish processing companies' financial return but also the reduction of the disposal of waste biomass and feedstock depletion. The concept of shell biorefinery has arisen within this context, referring to a new concept that aims for the fractionation of crustacean shells into their major components and their subsequent transformation into value-added products. However, due to the extensive covalent and hydrogen bonding between the different constituents of shells, their effective isolation can be a challenge.[1,8]

Received: June 29, 2021
Published: October 20, 2021

The most commonly used method for obtaining pure and colorless chitin from crustacean shell biomass involves the generation and disposal of large quantities of highly concentrated toxic chemicals into the aquatic ecosystem, including strong acids and bases, and does not allow the recovery of co-products, such as proteins or minerals.[9] Traditionally, this methodology involves a first demineralization step with strong acid at temperatures up to 100 °C and up to 2 days of reaction time, followed by deproteination with a strong base at temperatures up to 100 °C and up to 3 days of reaction time, and subsequent decoloration through oxidative bleaching.[8,10] Although there have been a lot of recent developments in the search for a more environmentally benign process for chitin extraction, namely biological methods, including enzymatic deproteination and fermentation using microorganisms, none of the methodologies proposed so far is competitive at commercial scales.[6,8,10] Therefore, there is a pressing need for the development of new technologies that allow a safer and cheaper chitin recovery.

Deep eutectic systems (DESs) have emerged in the last few years as viable alternatives to conventional solvents due to their remarkable solubilizing power toward very different molecules, including lipid- or poorly water-soluble compounds.[11−13] In particular, the application of different DESs in the fractionation of shrimp and lobster shells and in the recovery of chitin has already shown promising results.[3,14−17]

DESs are commonly defined as mixtures that undergo high melting point depressions in comparison to their neat components when prepared at certain ratios. These systems have shown to present several advantages when compared to widely used conventional solvents, namely the possibility of being easily scalable at low cost, or designed with acceptable toxicity, biodegradability, and biocompatibility features.[11] However, even though DESs have been generally pointed as being environmentally safe or nontoxic, it is important to consider potential interactions between the different components of the mixture, rather than focusing solely on the toxicity of the isolated compounds. Therefore, when envisioning the possible applications of DESs, the study of their toxicity is of the utmost importance.[12,18]

Within this context, in this work, low-phytotoxic DESs based on choline chloride (ChCl) and different organic acids, namely malonic, DL-malic, and DL-lactic acids, were investigated for the recovery of chitin from brown crab (*Cancer pagurus*) shell residues, a raw material that, to the best of our knowledge, remains unexplored in the existing literature. Accordingly, before being applied in extraction experiments, the phytotoxicity of the DESs prepared was assessed for the first time on wheat (*Triticum aestivum*) seeds by evaluating their impact on seedlings growth (inhibition of germination and shoots height), lipid peroxidation, photosynthetic pigments content, and antioxidant enzymes activity. After determining their phytotoxicity and aiming at maximizing the removal of minerals and proteins from the shells to yield pure chitin, ChCl/organic acid-based DESs were tested at different operating conditions, namely temperature (50, 80, and 130 °C) and processing time (2.5, 3, and 4 h), with and without the addition of water. The structure and properties of the obtained chitin samples were evaluated by different analytical techniques and were then compared to a standard as well as to chitin recovered from crab biomass through a conventional acid/alkaline hydrolysis (using hydrochloric acid, sodium hydroxide, ethanol, and acetone at temperatures up to 70 °C for a total processing time of 25 h).

RESULTS AND DISCUSSION

DES Preparation and *In Vitro* Phytotoxicity. DESs have been described by several authors as being nontoxic, green, and environmentally friendly. However, this assumption has been often based solely on the fact that DESs can be prepared from natural compounds, or alternatively, on the toxicity data already reported for each of their individual components. Still, it is important to note that DESs can have very different behavior when compared to their individual compounds, or even physical mixtures, due to differences in solubility or possible synergistic, additive, or antagonistic effects, which significantly influence their toxicity.[18−21] In addition, it has also been hypothesized that the charge delocalization that occurs through hydrogen bonding when a DES is formed can possibly lead to a more toxic system when compared to their individual components.[19] In this way, it is important to confirm the toxicological effect of a DES before envisioning a commercial application.[12,18]

DESs based on ChCl and different organic acids have already shown potential for the isolation of different polysaccharides, including chitin.[3,14−17,22] Therefore, in the work reported herein, ChCl was selected as a hydrogen bond acceptor, while malonic, DL-malic, and DL-lactic acids were selected as hydrogen bond donors for the DES formation and subsequent chitin recovery.

So far, the toxicity of the DES has been mainly assessed on microorganisms and cell models. However, after being used in an industrial process, the DES can be released into the soil, air, and water. Thus, it is of the utmost importance to know their effects at higher trophic levels (such as plants) since these effects cannot be extrapolated from lower levels of biological organization.[23] Accordingly, phytotoxicity assays have been commonly used to determine the capacity of a compound to cause temporary or long-lasting damage to plants[24] and can, therefore, help determine the environmental impact of a given system on terrestrial plants and crops. *T. aestivum* is a relevant crop (one of the most important economic crop plants worldwide), which has been frequently used as a reliable ecotoxicological indicator and model for phytotoxicity evaluation of environmental contaminants, pharmaceutical compounds, or nanomaterials.[25]

Within this context, to evaluate the impact of ChCl/organic acid-based DESs on wheat (*T. aestivum*), seeds were treated with a range of concentrations of each system for 7 days. Growth parameters, namely germination and shoot height inhibition, were measured and the corresponding half-maximal effective concentrations (EC50) were calculated.

Results show that both seed germination and growth were considerably inhibited by increasing concentrations of the DES studied (Figure S1). Additionally, results also suggest that the early growth of seedling shoots was more sensitive to the toxic effects of the DES than seed germination, which is in accordance with the work of Radošević et al. on the toxic impact of the DES based on mixtures of ChCl with glucose, glycerol, and oxalic acid on wheat seeds.[20] ChCl/lactic acid (1:1) showed the lowest toxicity, while ChCl/malonic acid (1:2) was the system that affected the most both germination and the shoot height, with EC50 ranging from 0.9 to 11.8 mg/mL, considering both markers (Table 1). It is also interesting to note that shoots grew in an uneven way in seeds treated

https://doi.org/10.1021/acsomega.1c03402
ACS Omega 2021, 6, 28729−28741

Table 1. EC50 Values Obtained on Wheat Seeds Treated with the ChCl/Organic Acid-Based DES, after an Incubation Period of 7 days

DES	EC50 (mg/mL)	
	germination	shoot height
ChCl/malonic acid (1:2)	5.0 ± 0.1	0.9 ± 0.1
ChCl/malic acid (1:2)	7.6 ± 1.9	1.3 ± 0.5
ChCl/lactic acid (1:1)	11.8 ± 0.3	1.6 ± 0.6

with the different systems when compared to the control, which was exacerbated as the concentrations of DESs increased. Nevertheless, no signs of leaf necrosis were observed (data not shown).

The phytotoxicity of ChCl-based DESs has already been explored by several authors on different model organisms, namely garlic (*Allium sativum*) cloves,[26] microalgae (*Raphidocelis subcapitata*),[27] and wheat (*T. aestivum*) seeds.[20] In particular, the work by Radošević et al. showed that, although the DES studied caused some degree of inhibition of seeds germination and shoot and root growth, the toxicity of the systems could still be regarded as low since the germination EC50 was superior to 5 mg/mL.[20] Therefore, taking into consideration the work of Radošević et al. and the toxicity classification proposed by Passino and Smith, in which chemicals are classified into several categories of toxicity based on their effective concentrations, it is possible to conclude that the DES studied were "relatively harmless" to wheat (EC50 > 1 mg/mL, the least toxic category according to this classification system).[27,28]

Oxidative stress is known to be related to germination and growth inhibition. In this way, the determination of lipid peroxidation (LPO) has been widely used as a marker of reactive oxygen species (ROS)-mediated damage, in particular by measuring the accumulation of malondialdehyde (MDA), the cytotoxic product of the peroxidation of unsaturated fatty acids contained in phospholipids.[29] The accumulation of MDA on the leaves harvested from seedlings after treatment with two different concentrations of DES (5 and 10 mg/mL) is presented in Figure 1i and shows that ChCl/malonic acid (1:2) was the only system that significantly increased the MDA content ($P < 0.0001$) at 10 mg/mL. These results are probably justified by the higher toxicity displayed by this system on germination and early growth of seedling shoots (EC50 of 5.0 and 0.9 mg/mL, respectively). Similar findings were reported for a ChCl/oxalic acid DES: the higher the concentration of the DES, the higher the MDA content, which shows an inability of the antioxidant enzymes to completely remove the accumulated ROS during DES treatment.[20]

The content of photosynthetic pigments [chlorophyll (CHL) *a*, *b*, and total CHL] in leaves has also been considered by several authors as an abiotic stress marker and as an indicator of plant health.[30] Generally, the CHL content did not show to be significantly affected by DES treatment (Figure 1ii), except when using ChCl/malonic acid (1:2) at 10 mg/mL ($P < 0.05$). Similarly to what was reported in previous works for ChCl/organic acid-based DESs,[20,27] these results correlate not only with the higher toxicity displayed by ChCl/malonic acid (1:2), showing an increased inhibition of the plants' growth, but also with an increase in the MDA content when treating wheat with this system at 10 mg/mL. This indicates that the accumulation of MDA may have contributed to the photosynthetic system damage, thus reducing photosynthesis, a key phenomenon that significantly contributes to the plant's growth and development under stress.[31]

Antioxidant enzymes such as superoxide dismutase (SOD), guaiacol peroxidase (GPX), catalase (CAT), and ascorbate peroxidase (APX), are part of a complex antioxidative defense system, which is of the utmost importance for plant survival and adaptation.[29] These and other enzymes are responsible for maintaining cell homeostasis and for providing a concerted response to oxidative stress.[29,32] Consequently, considering that plants are known to fine-tune their response to different stress factors, being the severity of stress symptoms highly

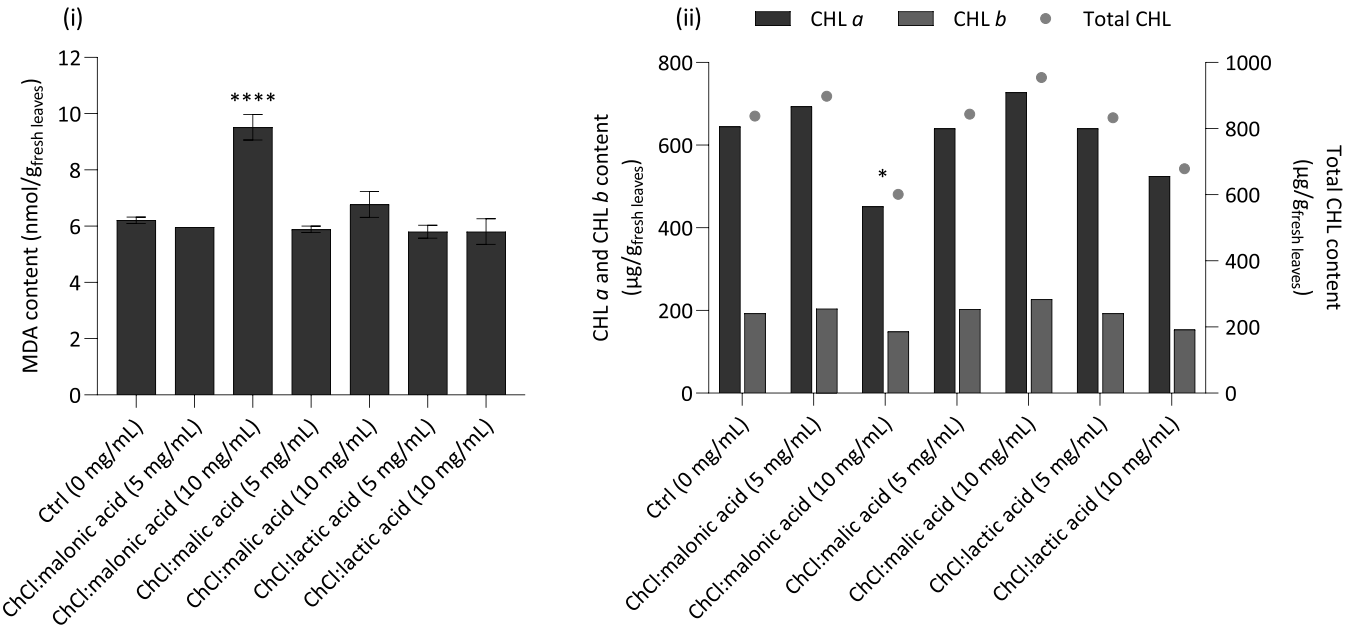


Figure 1. Effect of ChCl/organic acid-based DES on (i) MDA and (ii) the photosynthetic pigment content. Statistically significant differences between the effect of the DES and the control are represented by asterisks (*).*$P < 0.05$ and ****$P < 0.0001$.

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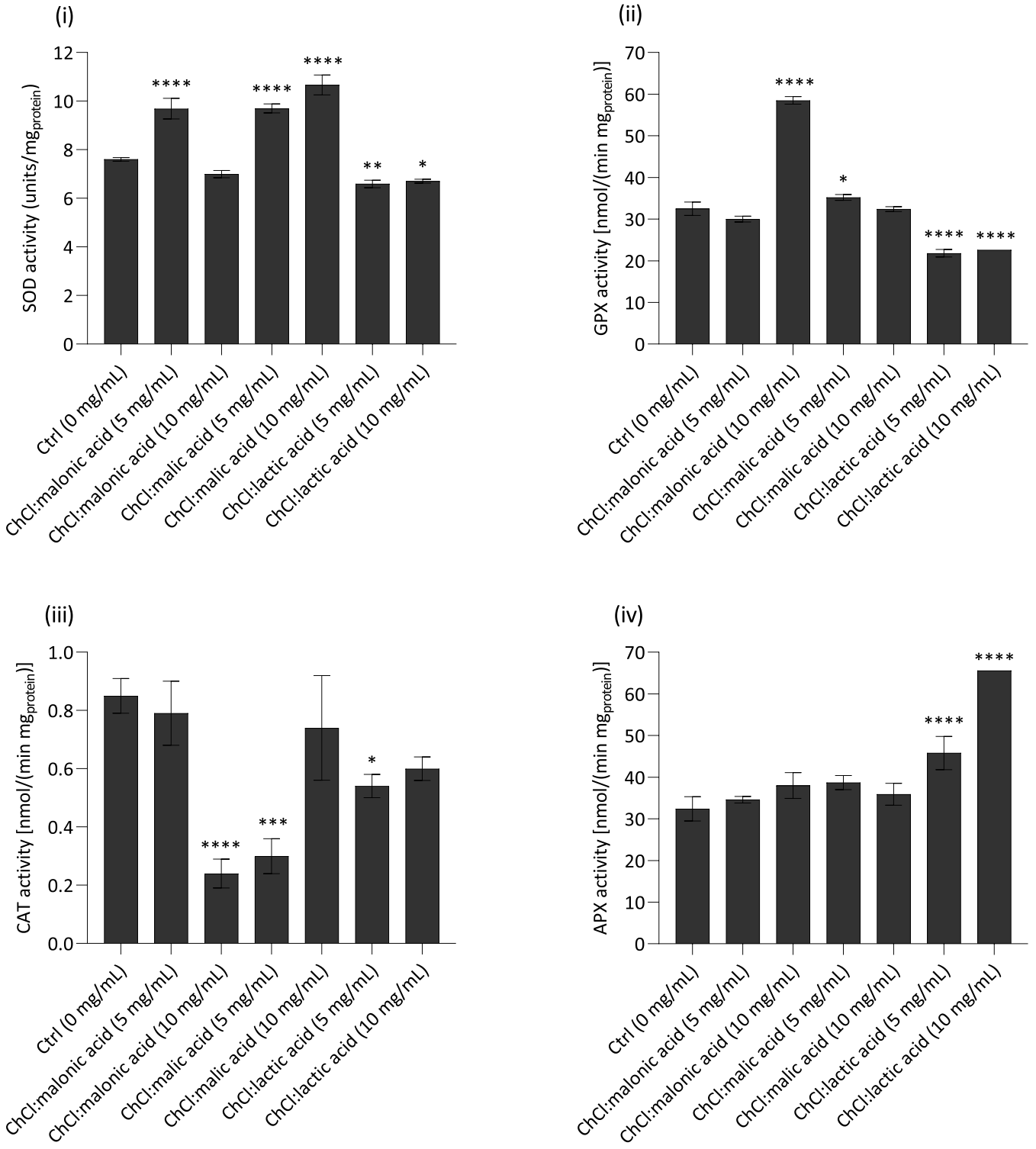

Figure 2. Effect of ChCl/organic acid-based DES on (i) SOD, (ii) GPX, (iii) CAT, and (iv) APX activities. Statistically significant differences between the effect of the DES and the control are represented by asterisks (*).*$P < 0.05$, **$P < 0.01$, ***$P < 0.001$, and ****$P < 0.0001$.

dependent on their capacity to tolerate a specific stress inducer, their up- or downregulation has been considered to be a good indicator of the oxidative stress level in the plant.[33,34]

Within this context, SOD, GPX, CAT, and APX were selected as biomarkers to determine the oxidative stress caused by the ChCl/organic acid-based DESs on the enzymatic antioxidant defense system of wheat. Figure 2 shows that, while some DES caused the activity of antioxidant enzymes to increase at specific concentrations, there were also cases where enzymes were slightly or fairly inhibited. This implies that the DES' toxicities were mostly affected by their specific characteristics and could eventually have been triggered by their hydrogen bond donors, which might have promoted the formation of different free radicals, such as singlet oxygen ($^1O_2$), superoxide radical ($O_2^{\bullet-}$), hydrogen peroxide (H_2O_2), or hydroxyl radical ($OH^{\bullet}$). The most toxic DES regarding

https://doi.org/10.1021/acsomega.1c03402
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germination and growth of seedlings, namely ChCl/malonic acid and ChCl/malic acid, showed a tendency to upregulate SOD and GPX while inhibiting CAT and maintaining APX activity. Conversely, the least-toxic DES ChCl/lactic acid caused downregulation of SOD, CAT, and GPX, while inducing APX activity. These results suggest that the defense mechanisms of wheat had a different antioxidant and redox homeostasis response to toxic levels of oxidative stress, depending not only on the system tested but also on the DES concentration applied and on the free radicals induced.

Chitin Recovery from the Crab Shell Biomass. Table 2 shows the proximate composition of brown crab shell residues.

Table 2. Proximate Composition of Freeze-Dried Brown Crab Shell Residues[a]

compound	g/100 $g_{dry\ residue}$
minerals	64.8
chitin	11.4
other compounds[b]	23.8

[a]Coefficient of variation ≤ 8.7%. [b]Mainly proteins and minor compounds (e.g., fatty acids, pigments).

As it would be expected, since the residues were mainly composed of shells, the most prominent component of the matrix was minerals (65%), followed by protein and other minor compounds (24%) and chitin (11%).

Although the relative percentage of each of these components is extremely dependent on the crab species and on seasonal changes, the results described in this work for minerals, proteins, and chitin were similar to those presented by other authors for both male and female *C. pagurus*, caught in Scottish and French waters.[35]

As previously mentioned, minerals, proteins, and chitin form a complex network, which can pose a challenge when the aim is to separate the different components of the crustacean's exoskeleton. In this way, for a successful chitin recovery, the solvent applied must be able to demineralize and deproteinize the shells. Demineralization usually requires acidic conditions, while high temperatures under acidic or alkaline conditions are crucial for deproteination so that proteins can be denatured.[3] Therefore, the traditional procedure for the fractionation of crustacean shell biomass involves the removal of minerals and proteins, and subsequent decoloration to obtain pure and colorless chitin.[8,10] Although this conventional process is extremely time-consuming and harmful to the environment,[8] it is in fact a highly efficient method to obtain pure chitin, as shown in Table 3. However, this process has resulted in considerable losses of chitin (approximately 16%) during the subsequent steps of extraction.

The preparation of ChCl/organic acid-based DES, as well as the operating conditions applied, was selected based on previous works, where these systems have proven to be effective in the extraction of chitin from shellfish biomass.[3,15−17]

In the first set of experiments, the DESs prepared were studied for the recovery of chitin at different operating temperatures for 4 h. However, as summarized in Table 3, DESs showed to be very ineffective in removing both minerals and proteins. Although, in general, efficiencies increased with temperature, probably facilitated by a decrease of DES viscosities, it was not possible to exceed 71% of demineralization (ChCl/malic acid (1:2) at 130 °C) or 88% of deproteination (ChCl/lactic acid (1:1) at 130 °C) efficiencies.

Therefore, the second set of experiments was designed, in which extractions were performed for 2 h with the selected DESs at different temperatures. After the first extraction step, water was added to the mixture, which was stirred for about 30 min, until room temperature was reached. The addition of water was expected to have a double function: on the one hand, it should cause the disruption of the DES structure, thus leading to the precipitation of any solubilized chitin; on the other hand, the presence of water should cause an acidic

Table 3. Operating Conditions, Hydrolysis Extent, and Demineralization and Deproteination Efficiencies for the Different DESs Tested

	temperature (°C)	processing time (h)	hydrolysis extent (%)	demineralization efficiency (%)	deproteination efficiency (%)[a]
conventional method	room temperature, 70	25	90.4 ± 0.4	100.0 ± 0.5	100.0 ± 0.1
ChCl/malonic acid (1:2)	50	4	45.2 ± 10.0	47.5 ± 5.7	60.5 ± 10.6
	80		52.1 ± 10.3	57.0 ± 8.4	63.9 ± 11.2
	130		50.8 ± 0.3	47.9 ± 1.7	83.0 ± 0.3
ChCl/malic acid (1:2)	50		29.1 ± 1.2	27.3 ± 2.6	47.8 ± 1.1
	80		31.3 ± 0.4	28.3 ± 2.9	54.4 ± 0.4
	130		65.6 ± 2.0	71.3 ± 5.8	81.5 ± 2.2
ChCl/lactic acid (1:1)	50		32.9 ± 3.1	31.4 ± 1.5	52.8 ± 3.0
	80		39.6 ± 0.1	40.1 ± 4.2	57.2 ± 0.1
	130		65.1 ± 8.9	68.0 ± 4.3	88.2 ± 9.3
ChCl/malonic acid (1:2)|H_2O	50	2|0.5	77.5 ± 1.7	99.7 ± 0.0	54.4 ± 2.1
	80		78.7 ± 1.8	100.0 ± 0.1	58.4 ± 2.3
	130		76.0 ± 3.2	92.3 ± 1.4	68.2 ± 3.9
ChCl/malic acid (1:2)|H_2O	50		75.7 ± 1.9	99.6 ± 0.9	47.0 ± 2.5
	80		76.8 ± 2.8	99.9 ± 0.0	50.7 ± 3.7
	130		81.1 ± 1.2	99.7 ± 0.8	69.5 ± 1.5
ChCl/lactic acid (1:1)|H_2O	50		72.4 ± 6.4	99.3 ± 1.0	33.9 ± 8.7
	80		74.3 ± 4.2	100.0 ± 0.5	40.1 ± 5.7
	130		85.1 ± 1.3	99.6 ± 0.0	86.7 ± 1.6

[a]Includes not only proteins but also other impurities (e.g., fatty acids, pigments).

https://doi.org/10.1021/acsomega.1c03402
ACS Omega 2021, 6, 28729−28741

Table 4. Operating Conditions, Hydrolysis Extent, Demineralization and Deproteination Efficiencies, and Chitin Purity for the Different DESs Tested at the Most Promising Temperature Conditions after Decoloration with Hydrogen Peroxide

	temperature (°C)	processing time (h)	hydrolysis extent (%)	demineralization efficiency (%)	deproteination efficiency (%)[a]	chitin purity (%)
ChCl/malonic acid (1:2)|H_2O|H_2O_2	80	2|0.5|0.5	84.2 ± 1.2	100.0 ± 0.2	81.9 ± 1.2	72.5 ± 1.1
ChCl/malic acid (1:2)|H_2O|H_2O_2	130		84.2 ± 0.8	99.7 ± 0.8	82.5 ± 0.8	72.4 ± 0.7
ChCl/lactic acid (1:1)|H_2O|H_2O_2	130		89.2 ± 1.5	99.7 ± 0.1	100.0 ± 0.5	98.2 ± 1.6

[a]Includes not only proteins but also other impurities (e.g., fatty acids, pigments).

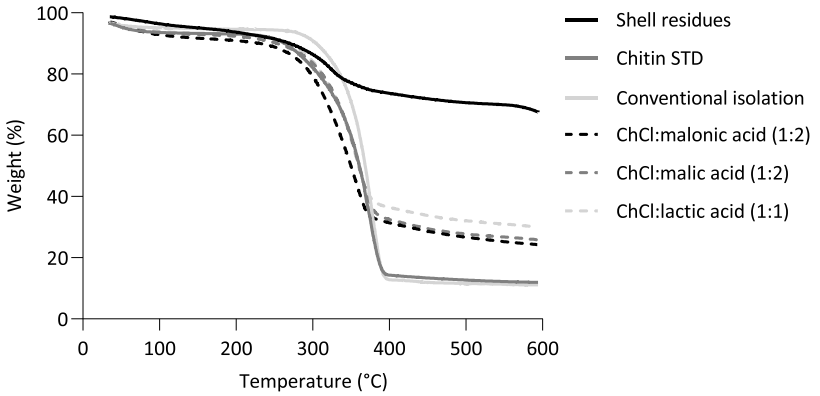

Figure 3. TGA curves of shell residues, chitin STD, and chitin recovered from the crab shell biomass by the conventional method and the ChCl/organic acid-based DES (obtained at the processing conditions summarized in Table 4).

environment through the formation of charged species, which was expected to improve the reaction of organic acids with minerals and further promote the acidic hydrolysis of proteins.[3] However, it is important to note that other authors have found that the addition of high water contents in the beginning of the extraction could negatively influence the removal of proteins.[17]

As expected, the results displayed in Table 3 show that the addition of water after the first extraction step was crucial to increase the demineralization efficiency to close to 100% for most of the conditions tested. However, deproteination was not as efficient as demineralization, ranging from around 34 to 87%, with ChCl/lactic acid (1:1) being the most promising system when used at 130 °C, as it had been observed for 4 h extraction. Nevertheless, it was possible to further enhance the deproteination efficiency during the decoloration step (Table 4), as hydrogen peroxide was able to remove the proteins that remained in the matrix, as previously reported by other authors.[36,37]

The fact that the ChCl/lactic acid DES outperformed ChCl/malonic acid and ChCl/malic acid in the deproteination of shells (up to 1.2-fold) might be related to the acidity of the hydrogen bond donor (malonic, malic, or lactic acids), as it was demonstrated by Zhou et al., through a linear positive correlation between the deproteination efficiency and the pK_a of the hydrogen bond donor.[38] Furthermore, it can also be hypothesized that this behavior might be related to the viscosity of each system (which increased as follows: ChCl/lactic acid < ChCl/malonic acid < ChCl/malic acid),[3,39] and to the consequent problems arising from limitations in mass transfer and diffusivity for more viscous systems.

Therefore, similarly to what was described for other biomass matrices,[3,16,38] by applying a methodology composed of the first step of demineralization and partial deproteination with ChCl/lactic acid (1:1) DES and water, followed by a step of decoloration and complete deproteination with hydrogen peroxide, it was possible to obtain high-purity chitin (98%) with fewer losses than those caused by the conventional hydrolysis (5% compared to 16% chitin losses). Furthermore, it can be of great benefit to use ChCl/lactic acid (1:1) as a solvent since, as previously discussed, this was the system that showed the least phytotoxicity on wheat seeds.

As shown in Table 2, shell residues still have a significant amount of minerals in their composition (around 65%). Therefore, and, if desirable, this mineral fraction can be isolated from the DES and eventually commercialized, either by precipitation with ethanol or sodium hydroxide, while the DES can be recycled and reused for further extractions.[3,17,40]

Chitin Characterization. The thermal stability of chitin is a critical factor when determining its potential applications. Thermogravimetric analysis (TGA) curves corresponding to crab shell residues, commercial chitin from shrimp shells (chitin STD), and chitin samples recovered from shell biomass by the conventional method or the ChCl/organic acid-based DES (obtained at the processing conditions summarized in Table 4) are presented in Figure 3. A first slight mass loss, which was common to all samples, could be perceived between 35 and 100 °C, possibly due to the evaporation of chemisorbed water.[15,16,41] The second stage of degradation between 100 and 250 °C was followed, being mainly noticeable in crab shells, which was probably due to the breakdown of proteins and lipids. The absence of an evident mass loss at this temperature range in the chitin samples recovered with the DES or by conventional hydrolysis helps to confirm that proteins were removed from the matrix.[38,41,42] As a result of chitin degradation, a third abrupt decomposition step could be noticeable between 250 and 400 °C.[15,16,38,40−42]

The thermal stability of a given material can be inferred by looking at the initial decomposition temperature. As shown in Figure 3, the stability of the chitin samples obtained after DES treatment was very similar to the chitin STD, whereas the stability of the chitin obtained by conventional hydrolysis was

https://doi.org/10.1021/acsomega.1c03402
ACS Omega 2021, 6, 28729−28741

superior. This may be related to the molecular weights of the different fractions of chitin, suggesting that the chitin recovered with the DES and the standard have similar molecular weights, which were probably lower than the molecular weight of the chitin obtained by conventional acid/alkaline hydrolysis. It is possible that the higher temperatures applied during DES treatment resulted in increased hydrolysis of chitin, consequently leading to macromolecules with a lower molecular weight.[40]

The Fourier transform infrared-attenuated total reflection (FTIR-ATR) spectra of the crab shells, chitin STD, and chitin recovered from the shell biomass by acid/alkaline hydrolysis and the DES (obtained at the processing conditions summarized in Table 4) are shown in Figure 4. It is interesting

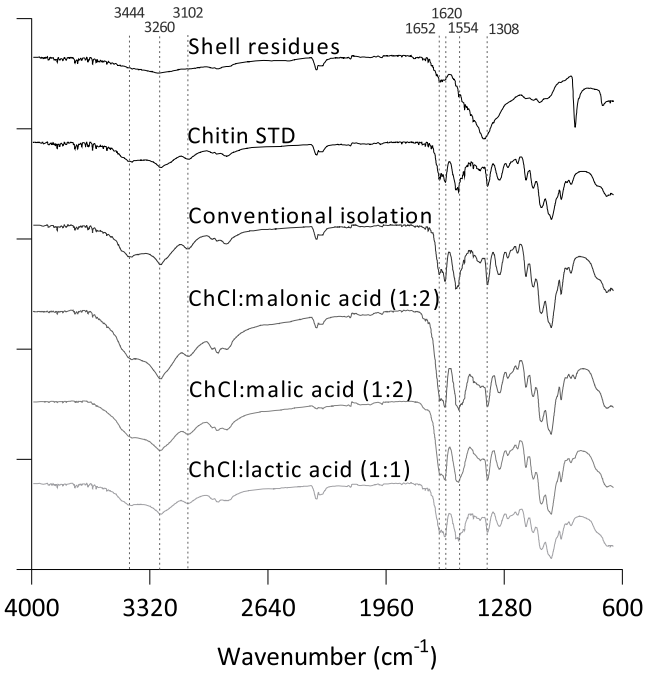

Figure 4. FTIR-ATR spectra of shell residues, chitin STD, and chitin recovered from the crab shell biomass by the conventional method and the ChCl/organic acid-based DES (obtained at the processing conditions summarized in Table 4).

to note that, except for crab shells, all samples showed very similar patterns, presenting all typical absorption bands of chitin STD. The assignments of chitin absorption peaks were according to the previous literature,[15,16,38,40−42] namely the symmetric stretching vibration of O−H at 3444 cm^{-1} and N−H at 3260 and 3102 cm^{-1}; the amide I band split at 1652 and 1620 cm^{-1}, attributed to the presence of intermolecular (−CO···HN−) and intramolecular (−CO···$HOCH_2$−) hydrogen bonds; the amide II band at 1554 cm^{-1} attributed to in-plane N−H bending and C−N stretching; and the amide III band at 1308 cm^{-1} attributed to the C−H bend.

Regarding the spectrum of crab shells, it is possible to conclude from Figure 4 that the chitin amide I band was not clearly split due to the overlapping of the protein amide peaks.[41,42] This suggests that both conventional hydrolysis and the ChCl/organic acid-based DES were able to remove proteins from the shell matrix.

It is worth highlighting that the deacetylation degree of samples obtained after DES treatment was lower than 6.4%, with the acetylation degree increasing as follows: ChCl/malic acid (93.6%) < ChCl/malonic acid (96.8%) < chitin STD = conventional acid/alkaline hydrolysis (98.0%) < ChCl/lactic acid (98.5%).

To evaluate the crystal structure and crystallinity of samples, powder X-ray diffraction (XRD) analysis was performed on crab shell residues, chitin STD, and on the chitin samples recovered from the shells by the conventional method and ChCl/organic acid-based DES (obtained at the processing conditions summarized in Table 4). The profiles illustrated in Figure 5 show that the chitin samples recovered with DES are

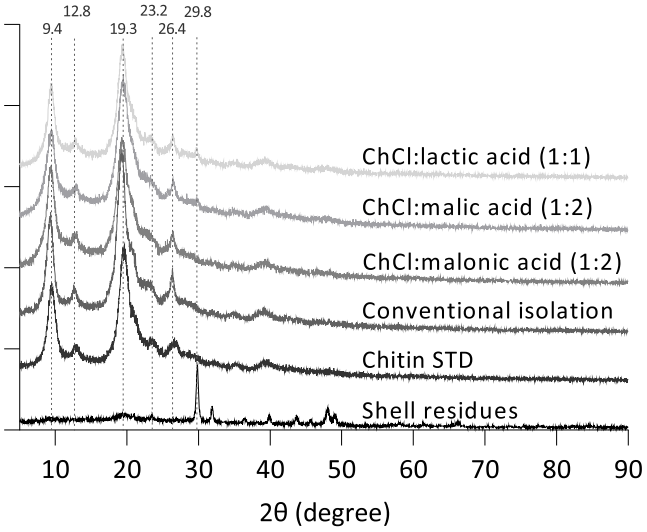

Figure 5. XRD profiles of shell residues, chitin STD, and chitin recovered from the crab shell biomass by the conventional method and the ChCl/organic acid-based DES (obtained at the processing conditions summarized in Table 4).

in good agreement with what was obtained for the chitin STD or for chitin recovered using the conventional method. Unlike shell residues, all chitin samples displayed two main diffraction peaks at $2\theta \approx 9.4$ and 19.3°, and three weaker diffraction peaks at $2\theta \approx 12.8$, 23.2, and 26.4°, which are characteristics of the crystalline structure of α-chitin.[15,16,38,41] When comparing the diffraction patterns displayed by chitin with the pattern of shells, it is interesting to note that the diffraction peak at $2\theta \approx 29.8°$, characteristic of calcium carbonate,[40−42] decreased in chitin samples recovered either by the conventional method or by the DES, while the peaks characteristic of α-chitin increased. This suggests that the chitin concentration increased as calcium carbonate was removed.

Crystallinity indexes of crab shell residues, chitin STD, and chitin samples recovered by acid/alkaline hydrolysis, ChCl/malonic acid, ChCl/malic acid, and the ChCl/lactic acid DES, were as follows: 43.0, 84.5, 85.7, 78.6, 79.2, and 82.9%. The increase of chitin crystallinity when compared to the shells suggests that both minerals and proteins were successfully removed from the matrix.[40−42] Furthermore, the ChCl/lactic acid DES was able to produce chitin with a crystallinity index close to those of chitin STD or chitin obtained by conventional hydrolysis.

Scanning electron microscopy (SEM) images of the shell residues, chitin STD, and chitin recovered after conventional acid/alkaline hydrolysis and DES treatment (obtained at the processing conditions summarized in Table 4) are presented in Figure 6. It is possible to conclude from SEM observations that

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ACS Omega 2021, 6, 28729−28741

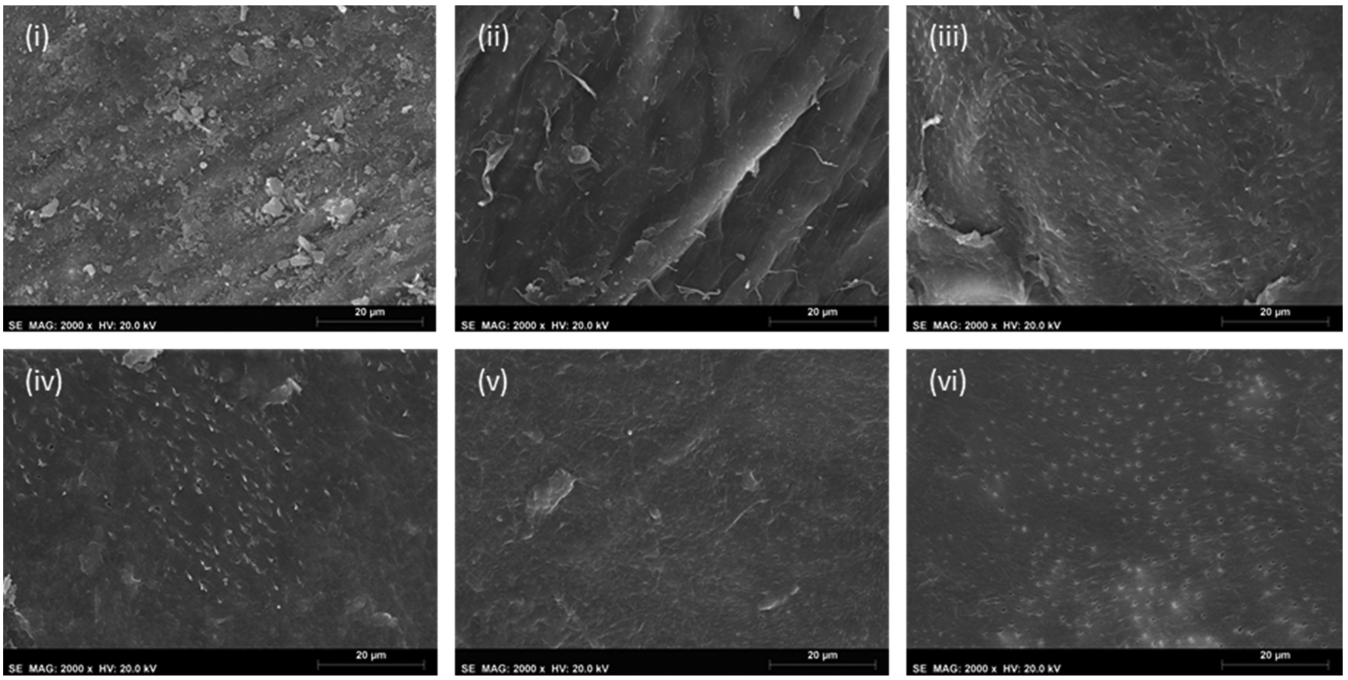

Figure 6. SEM images of (i) shell residues, (ii) chitin STD, and chitin recovered from the crab shell biomass by (iii) conventional hydrolysis, (iv) ChCl/malonic acid (1:2), (v) ChCl/malic acid (1:2), and (vi) ChCl/lactic acid (1:1) treatment (obtained at the processing conditions summarized in Table 4).

there was a considerable modification of the surface of the recovered chitin samples when compared to those of crab shells or the standard. A rough surface without pores was observed for the shells as well as for the standard (Figure 6i,ii, respectively), while smoother surfaces with pores were visible in chitin recovered with the conventional method or with the DES due to mineral and protein removal (Figure 6iii–vi). It is interesting to note that, as reported by other authors, chitin presenting a porous surface structure may be interesting for applications such as the adsorption of metal ions or dyes.[15,38,40]

Although the particle size of the standard was revealed to be higher than the remaining materials, the particle size of the chitin samples obtained after different treatments was quite similar to the size of the crab shell particles (Figure S2), confirming that the intrinsic structure of chitin was not degraded into smaller particles. These results are in accordance with the work developed by Zhou et al., in which chitin was isolated from black soldier flies with ChCl/lactic acid and betaine/urea DES.[38]

CONCLUSIONS

The study reported herein demonstrated an alternative approach for chitin recovery from the brown crab shell biomass, using the low-phytotoxic ChCl/organic acid-based DES, which has the potential to become competitive at commercial scales.

Overall, DESs have shown low toxicity to wheat seeds, with EC50 ranging from 5 to 12 mg/mL considering germination and from 0.9 to 1.6 mg/mL considering shoot height, with ChCl/malonic acid being the most toxic and ChCl/lactic acid the least toxic DES.

Due to the multifunctional properties of these systems, the shell residues could be demineralized, deproteinized, and decolored to yield chitin in just 3 h (in a three-step process using the DES, water, and hydrogen peroxide), as opposed to the 25 h necessary to obtain pure chitin through conventional hydrolysis (in a four-step process using hydrochloric acid, sodium hydroxide, ethanol, and acetone, at temperatures up to 70 °C). ChCl/lactic acid (1:1) was revealed to be the most interesting system for chitin recovery at 130 °C, resulting in the highest demineralization and deproteination efficiencies. Minerals were mainly removed by the acidic conditions provided by the DES and the added water, while proteins could be removed, in a first stage, by the high temperatures applied, and in a second stage by hydrogen peroxide, whose main function was the removal of pigments and other impurities that remained in the recovered chitin.

The characterization experiments performed on chitin obtained by the ChCl/lactic acid DES treatment have shown that it was possible to obtain chitin with features similar to a commercial product or to chitin obtained by conventional hydrolysis, namely similar thermal stability, degree of acetylation, and crystallinity. Furthermore, the chitin obtained after DES treatment was revealed to have a porous surface structure, which might enable its application in market sectors such as adsorption of metal ions or dyes, that would not be viable if chitin presented a nonporous structure.

It is also interesting to note that it has already been demonstrated by other authors that it is possible to recover not only the calcium carbonate extracted from the shells while using DESs as solvents, but also to recycle and reuse the DESs, being a very important feature from an environmental and economic point of view.

METHODS

Raw Material. Brown crab (*C. pagurus*) shells were kindly provided by Tejo Ribeirinho, Portugal, in November 2017, and stored at −20 °C upon arrival, in the absence of light. Before the extraction experiments, the residues were dehydrated using a Coolsafe Superior Touch 55-80 freeze dryer (Scanvac,

https://doi.org/10.1021/acsomega.1c03402
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Labogene, Bjarkesvej, Denmark) at −55 °C for approximately 72 h. Freeze-dried shells were then milled using a cutter-emulsifier CKE-8 (Sammic, Azkoitia, Gipuzkoa, Spain), and the particle size of the ground material was determined (measuring range: 250−710 μm) using an AS 200 basic vertical vibratory sieve shaker (Retsch, Haan, Germany). After processing, the residues were protected from light and stored at room temperature in a low-moisture environment until the day of experiments.

DES Preparation. ChCl (ref C7527) and DL-malic acid (ref 240176) from Sigma-Aldrich (China), malonic acid (ref A11526) from Alfa Aesar (Kandel, Germany), and DL-lactic acid (ref 125065000) from Acros Organics were used for the DES preparation. Systems were prepared by heating the mixture of the two components to 80 °C, in the case of ChCl/malonic acid and ChCl/lactic acid, and 90 °C in the case of ChCl/malic acid, under constant stirring, until a clear liquid was formed. The mixtures were then allowed to cool to room temperature before being used in characterization or extraction experiments.

***In Vitro* Phytotoxicity Determination of the DES.** *Sample Preparation.* Stock solutions of DESs were prepared in distilled water immediately before the phytotoxicity assay. Samples were then 2-fold serially diluted in distilled water to obtain a range of concentrations (0−20 mg_{DES}/mL).

Phytotoxicity Assay. The phytotoxicity of DESs was determined according to the methods of Cvjetko Bubalo et al. and Radošević et al.[20,31] Briefly, wheat (*T. aestivum*) seeds were sterilized for 30 min using a solution of 1% (v/v) sodium hypochlorite (Sigma-Aldrich, St. Quentin Fallavier, France). Seeds were then washed several times with distilled water and incubated for 24 h in the darkness at 23 ± 1 °C. After incubation, 15 seeds were placed in each Petri dish (Ø = 15 cm), previously prepared with two pieces of filter paper covered by a thin layer of cotton wool, and moistened with 30 mL of different DES concentrations. Control was maintained with distilled water. Wheat seedlings were grown for 7 days at 23 ± 1 °C with shift cycles of 14 h/day and 10 h/night. To keep DES concentrations stable, seeds were re-moistened with each solution every 48 h. After a 7 day treatment, seedlings were harvested and the effect of DESs on germination and early growth of wheat was determined. All experiments were performed in duplicates. Results were expressed as germination inhibition and shoot height inhibition in comparison to controls. EC50 values were calculated from dose−response curves and expressed as mg_{DES}/mL.

LPO Determination. LPO was determined by homogenizing approximately 0.2 g of fresh leaves in 5 mL of 0.1% (m/v) trichloroacetic acid (Fisher Scientific, Loughborough, United Kingdom), using a mortar and pestle. LPO levels were measured by the determination of MDA content in the supernatants, using the thiobarbituric acid (TBA) method.[43] Briefly, mixtures containing 1 mL of supernatant and 4 mL of 0.5% (m/v) TBA (Acros Organics, Geel, Belgium) in 20% (m/v) trichloroacetic acid were prepared and heated to 95 °C for 30 min. The mixtures were then cooled in an ice bath and centrifuged for 20 min at 5000*g*. Absorbances were measured at 532 nm using a GENESYS 10S UV−vis spectrophotometer (Thermo Fisher Scientific, Waltham, MA) and corrected for unspecific turbidity by subtracting the absorbance of samples at 600 nm. The MDA content was calculated according to its molar extinction coefficient (ε = 155 mM^{-1} cm^{-1}) and expressed as $nmol_{MDA}/g_{fresh\ leaves}$ as a mean of at least two measurements.

CHL Content Determination. The CHL content was determined using the method previously described by Arnon.[44] Briefly, approximately 0.1 g of fresh leaves were extracted with 10 mL of 80% (v/v) acetone (eci, Zagreb, Croatia) for 24 h at 4 °C under constant stirring. Samples were then centrifuged at 5000*g* and 4 °C for 15 min, and the supernatant absorbance was measured at 663 nm and 645 nm. The contents of CHL *a*, CHL *b*, and total CHL were calculated using eqs 1−3, respectively.

$$\text{CHL } a = 12.25A_{663} - 2.79A_{645} \quad (1)$$

$$\text{CHL } b = 21.50A_{645} - 5.10A_{663} \quad (2)$$

$$\text{total CHL} = \text{CHL } a + \text{CHL } b \quad (3)$$

Results were expressed as $\mu g_{CHL}/g_{fresh\ leaves}$.

Antioxidant Enzyme Activities. Supernatants used for both the protein content and the antioxidant enzyme activity determination were prepared by homogenizing in a prechilled mortar and pestle approximately 0.4 g of fresh leaves with 0.2 g of hydrated poly(vinylpolypyrrolidone) (Acros Organics, China) in 4 mL of 100 mM potassium phosphate buffer solution (pH 7.0; potassium phosphate monobasic, Fisher Scientific, Loughborough, United Kingdom; potassium phosphate dibasic, Kemika, Zagreb, Croatia), containing 1 mM ethylenediaminetetraacetic acid (EDTA, LKB Bromma, Stockholm, Sweden) and 5 mM L-ascorbic acid (Kemika, Zagreb, Croatia). Extracts were centrifuged at 5000*g* and 4 °C for 20 min, and the supernatants were recovered.

The total soluble protein contents were estimated according to the method proposed by Bradford,[45] using bovine serum albumin (Sigma-Aldrich, St. Quentin Fallavier, France) as standard. The experiments were performed in duplicates.

SOD activity was determined by measuring the inhibition of the photochemical reduction of nitro blue tetrazolium, as previously described by Beauchamp and Fridovich.[46] Briefly, the reaction was prepared in test tubes by mixing potassium phosphate buffer (50 mM, pH 7.8), EDTA (0.1 mM), methionine (13 mM, Acros Organics, China), nitro blue tetrazolium chloride (75 mM, Alfa Aesar, Kandel, Germany), riboflavin (2 mM, Carlo Erba Reagents, Val de Reuil, France), and a suitable aliquot of enzyme extract. The mixture was homogenized, and tubes were placed 30 cm away from a 30 W fluorescent lamp. The increase in absorbance due to formazan formation was measured immediately at 560 nm. The activity of one unit of SOD was defined as the amount of enzyme that inhibited the nitroblue tetrazolium photoreduction by 50%. The activity of SOD was expressed as $units_{SOD}/mg_{protein}$ as a mean of two measurements.

GPX activity was estimated by preparing a reaction mixture containing phosphate buffer (50 mM, pH 7.0), guaiacol (18 mM, Acros Organics, China), hydrogen peroxide (5 mM, 30%, Gram mol, Zagreb, Croatia), and a suitable aliquot of enzyme extract, and measuring the increase in absorbance of oxiguaiacol at 470 nm (ε = 26.6 mM^{-1} cm^{-1}), as previously reported by Chance and Maehly.[47] Experiments were performed in duplicates and results were expressed as $nmol_{oxidized\ guaiacol}/(mg_{protein}\ min)$.

CAT activity was determined as previously described by Aebi.[48] The reaction mixture was prepared with phosphate buffer (50 mM, pH 7.0), hydrogen peroxide (10 mM), and a suitable aliquot of the enzyme extract, and the decomposition

https://doi.org/10.1021/acsomega.1c03402
ACS Omega 2021, 6, 28729−28741

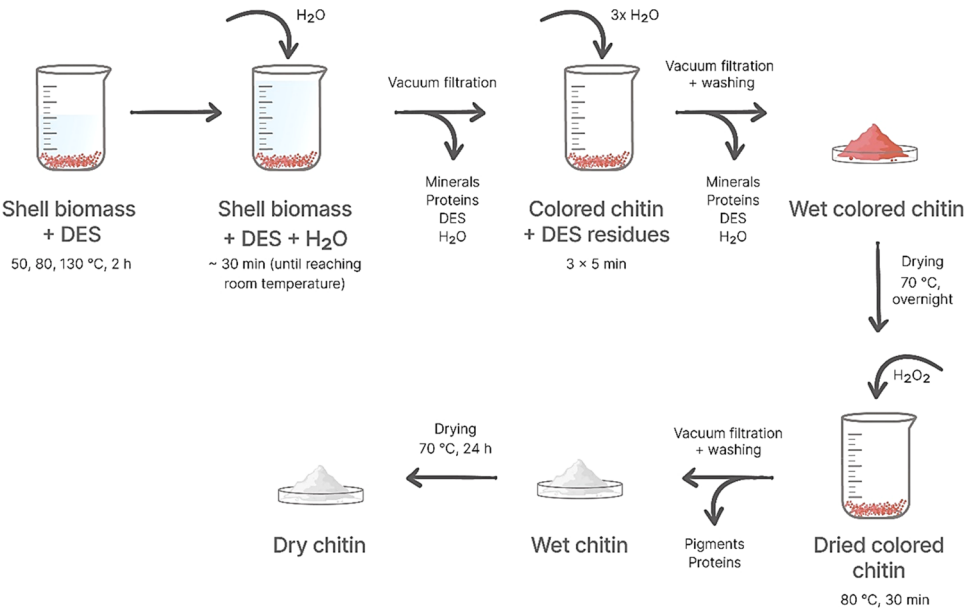

Figure 7. Schematic illustration of the second set of experiments for chitin recovery with DES.

of hydrogen peroxide and consequent decrease in absorbance at 240 nm was evaluated immediately (ε = 40 mM^{-1} cm^{-1}). Results were expressed as $nmol_{decomposed\ H_2O_2}/(mg_{protein}\ min)$ as a mean of at least two measurements.

APX activity was measured according to the method described by Nakano and Asada.[49] The reaction mixture contained phosphate buffer (50 mM, pH 7.0), EDTA (0.1 mM), L-ascorbic acid (0.5 mM), hydrogen peroxide (0.12 mM), and a suitable aliquot of the enzyme extract. Experiments were performed in duplicates by following the decrease in absorbance of ascorbate at 290 nm (ε = 2.8 mM^{-1} cm^{-1}), and results were expressed as $nmol_{oxidized\ ascorbate}/(mg_{protein}\ min)$.

Proximate Composition Analysis of the Crab Shell Biomass. The total ash content in freeze-dried brown crab shells was determined as described in Norma Portuguesa NP2032.[50] Briefly, crucibles containing a given amount of dried residue were placed in a muffle furnace at 550 °C for 6 h. The total mineral content was calculated through mass differences and results were expressed as $g_{minerals}/100\ g_{dry\ residue}$.

The total chitin content was determined as previously reported by Bradić et al.[3] Briefly, 50 mL of 1 M hydrochloric acid (≥37%, Sigma-Aldrich, Austria) was added to 0.4 g of freeze-dried residue and heated to 105 °C. After 1 h incubation, the mixture was filtered under vacuum and the residue was washed with distilled water until attaining a neutral pH. The remaining solid was further extracted with 100 mL of a 5% sodium hydroxide (Acros Organics, Sweden) solution and heated to 105 °C. After 1 h incubation, the mixture was filtered under vacuum and the residue was washed with distilled water until achieving a neutral pH. The remaining solid was further washed with 30 mL of acetone (Fisher Chemical, Loughborough, U.K.). The resulting solid samples were dried in an oven at 110 °C until a constant weight was achieved, after which they were incinerated in a muffle furnace at 600 °C for 6 h. The total chitin content was calculated through the mass loss during the incineration process, and results were expressed as $g_{chitin}/100\ g_{dry\ residue}$.

The total protein content (which also included other minor compounds, such as fatty acids, pigments, etc.) was determined by mass balance considering the results obtained for minerals and chitin, as previously described by Pires et al.[35] Results were expressed as $g_{protein}/100\ g_{dry\ residue}$.

All experiments were performed in duplicates.

Chitin Recovery from the Crab Shell Biomass. *Conventional Extraction.* Conventional hydrolysis of chitin was performed according to Al Sagheer et al. following three sequential steps: demineralization with strong acid, deproteination with a strong base, and decoloration with ethanol and acetone.[51] Briefly, demineralization was carried out with 0.25 M hydrochloric acid at a solid/liquid ratio of 1:40 g/mL for 90 min at room temperature. The acid was removed by decantation and this procedure was repeated three times. The resulting solids were washed with distilled water until neutral pH was attained and dried at 70 °C for 20 h before being used in deproteination experiments. Deproteination was performed using 1 M sodium hydroxide at a solid/liquid ratio of 1:20 g/mL for 30 min at 70 °C. The treatment was repeated three times, the last treatment being left overnight. The sodium hydroxide solution was removed by decantation and the resulting solids were washed to neutrality. To remove any impurities, such as pigments, the solids were washed with ethanol (Carlo Erba, Val de Reuil, France) at 70 °C and a solid/liquid ratio of 1:10 g/mL. After removing the ethanol, the remaining solids were boiled in acetone. The purified chitin was dried at 70 °C for 24 h, and then stored at room temperature, in a low-moisture environment, until further analyses. Mineral, protein, and chitin contents were determined through mass differences throughout the different extraction steps.

DES Extraction. The recovery of chitin using DESs was performed as previously described by Saravana et al.,[16] with slight modifications. As a first approach, approximately 12.5 g of the DES was added to 0.5 g of freeze-dried residue. Extractions were carried out in silicon baths for 4 h at 50, 80, and 130 °C, under constant stirring (ca. 60 rpm). The resulting extracts were filtered under vacuum and the solids were

https://doi.org/10.1021/acsomega.1c03402
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washed with distilled water until a neutral pH was attained. The solids were recovered and dried overnight at 70 °C.

The second set of extractions were conducted for 2 h, while maintaining the solid/liquid ratio, temperatures, and stirring rate as mentioned above. Distilled water was then added to the resulting extracts at a solid/liquid ratio of 1:25 g/mL (in relation to the initial mass of shell residue), and samples were stirred until reaching room temperature (approximately 30 min). The supernatant was filtered under vacuum and the solids were washed with distilled water, allowing 5 min of contact time, with occasional manual stirring. This procedure was repeated twice. Samples were then filtered under vacuum and the solids were repeatedly washed with distilled water until reaching a neutral pH. The solids were recovered, dried overnight at 70 °C, and the most promising samples were subjected to decoloration with hydrogen peroxide (30%, Carlo Erba, Val de Reuil, France), at a solid/liquid ratio of 1:10 g/mL and 80 °C for 30 min, with manual agitation every 10 min. The resulting solids were washed with distilled water and dried in an oven for 24 h at 70 °C. Figure 7 schematically illustrates the process.

Dried samples were stored at room temperature, in a low moisture environment, until further analysis. The hydrolysis extent was calculated as follows

$$\text{hydrolysis extent (\%)} = \frac{\text{initial mass of feed} - \text{final mass of feed}}{\text{initial mass of feed}} \times 100 \quad (4)$$

All experiments were performed at least in duplicate.

Chitin Characterization. The ash content of the dried solids was measured using a muffle furnace at 550 °C for 5 h, while the protein content was determined by mass balance considering the results obtained for minerals and chitin content. Demineralization and deproteination efficiencies were calculated according to eqs 5 and 6, respectively.

$$\text{demineralization efficiency (\%)} = \frac{\text{initial mass of minerals} - \text{final mass of minerals}}{\text{initial mass of minerals}} \times 100 \quad (5)$$

$$\text{deproteination efficiency (\%)} = \frac{\text{initial mass of proteins} - \text{final mass of proteins}}{\text{initial mass of proteins}} \times 100 \quad (6)$$

The purity of the obtained chitin was calculated according to eq 7.

$$\text{chitin purity (\%)} = \frac{\text{final mass of chitin}}{\text{final mass of feed}} \times 100 \quad (7)$$

TGA was performed using a Q50 thermogravimetric analyzer (TA Instruments, New Castle, DE) from 35 to 600 °C, at 10 °C/min, under a nitrogen atmosphere.

FTIR-ATR analyses were carried out using a Thermo Scientific FTIR spectrometer (Class 1 Laser Product Nicolet 6100, San Jose, CA). The equipment included ATR accessories with a diamond crystal of 42°. Spectra were recorded at room temperature between 4000 and 650 cm^{-1}. A background spectrum was recorded before acquisition and used as a reference. The final spectrum corresponds to the average of 32 individual scans, obtained with a resolution of 4 cm^{-1}. The degree of acetylation of chitin samples was determined as previously described by Kasaai, using the absorbance obtained at 1560 cm^{-1} as the intensity of a probe band and the absorbance obtained at 1160 cm^{-1} as the intensity of a reference band.[52]

XRD spectra were recorded on a Miniflex II XRD (Rigaku, Tokyo, Japan) operated at 30 kV and 15 mA with Cu/Kα as a radiation source, in the 2θ range of 5−90°. Spectra were recorded at room temperature, at a scanning rate of 5 °/min. The crystallinity index (CrI) was calculated as suggested by Segal et al.[53] according to eq 8

$$\text{CrI (\%)} = \frac{I_{110} - I_{am}}{I_{110}} \times 100 \quad (8)$$

where I_{110} is the maximum intensity of the diffraction peak at $2\theta \approx 19°$ and I_{am} is the intensity of amorphous diffraction at $2\theta \approx 16°$.

The surface morphology of crab shells and chitin samples was examined using an SEM (S2400, Hitachi High Technologies, Tokyo, Japan), operated at an acceleration voltage of 20 kV. Prior to imaging, samples were prepared on metal stubs, using an electrically conductive double-sided adhesive tape and then coated with a gold/palladium thin film using a sputter coater (Quorum Technologies, QT150T ES, Lewes, U.K.).

All characterization experiments were also performed on chitin from shrimp shells (coarse flakes, 98.0% acetylated, Sigma-Aldrich, Iceland) for comparison purposes.

Statistical Analysis. The estimation of phytotoxicity, as well as the statistical significance of average differences determination, was performed using GraphPad Prism 9 software (GraphPad Software, Inc., La Jolla, CA). The statistical significance of average differences was assessed by one-way ANOVA followed by the Tukey test. An alfa error of 5% was accepted in the hypothesis testing to decide for a significant effect. Data were reported as mean ± standard deviation values.

■ ASSOCIATED CONTENT

Supporting Information

The Supporting Information is available free of charge at https://pubs.acs.org/doi/10.1021/acsomega.1c03402.

Dose−response curves of the phytotoxic effect induced by the ChCl/organic acid-based DES evaluated on wheat seeds; and SEM images of shell residues, chitin STD, and chitin recovered from the crab shell biomass (40× magnification) (PDF)

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https://doi.org/10.1021/acsomega.1c03402
ACS Omega 2021, 6, 28729−28741

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Notes

The authors declare no competing financial interest.

■ ACKNOWLEDGMENTS

This work was financed by Portugal 2020 through the European Regional Development Fund (ERDF) in the frame of Operational Competitiveness and Internationalization Program (POCI) in the scope of the project MultiBiorefinery—POCI-01-0145-FEDER-016403, co-financed by national funds through the FCT/MEC. Additional funding was received from PTDC/EQU-EQU/29851/2017 project, financed by national funds (FCT/MEC); and from ERC-2016-CoG 725034 grant agreement, financed by the European Union's Horizon 2020 (European Research Council). This work was also supported by the funding received from INTERFACE Program, through the Innovation, Technology and Circular Economy Fund (FITEC); and from iNOVA4-Health (UIDB/04462/2020) and the Associate Laboratory for Green Chemistry—LAQV (UIDB/50006/2020), programs financially supported by FCT/MEC, through national funds. This work has also received support from COST Action CA18224 through an STSM Grant. Liliana A. Rodrigues, Alexandre Paiva, and Ana A. Matias have also received financial support from FCT through the SFRH/BD/116002/2016, IF/01146/2015, and IF/00723/2014 grants, respectively. The authors are grateful to Tejo Ribeirinho Lda. for kindly supplying the biomass used in this work.

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https://doi.org/10.1021/acsomega.1c03402
ACS Omega 2021, 6, 28729−28741