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Green extraction of chitin from hard spider crab shells

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ABSTRACT

A green protocol to extract chitin from crab shells using water soluble ionic liquids (ILs) is here reported. Compared to conventional multistep acid-base extraction methods, this one-pot procedure achieves pulping of recalcitrant crustacean waste shells by employing ammonium acetate, ammonium formate and hydroxylammonium acetate as water-soluble, low-cost and easy to prepare ILs. An extensive parametric analysis of the pulping process has been carried out with different ILs, different ratios, temperature and time. The optimized protocol provides a high-quality chitin comparable, if not better, to commercial chitin. The best results were obtained at 150 °C with ammonium formate prepared in-situ from aqueous ammonia and formic acid: chitin was isolated in a 17 wt% yield (based on dried crab shells as starting biowaste), a degree of acetylation (DA) > 94 %, a crystallinity index of 39–46 %, a molecular weight up to 6.6×10^5 g/mol and a polydispersity of ca 2.0.

1. Introduction

According to the Food and Agriculture Organization (FAO) fishery and aquaculture production will reach 202 million tonnes/year by 2030 (Maschmeyer et al., 2020). This sector is recognized in the context of food security and nutrition by the UN 2030 Agenda for Sustainable Development. A consequence of the increasing consumption of fishery-derived food is the production of waste, which is currently estimated in the order of dozens of million tons per year. Fishery waste represents a valuable source of chemical richness as it includes oils (e.g. ω-3 fatty acids), as well as amino acids, bioactive peptides, collagen, chitin, gelatin, and pigments (Kerton et al., 2013; Maschmeyer et al., 2020). Therefore, developing processes and technologies for the recovery and upgrading of such waste offers significant opportunities within the paradigm of the circular economy. Not only would this provide marketable products, but it would also contribute to reducing the effect of the anthropic exploitation of marine resources and to preserve coastal environments.

Crustacean shell biorefining is expected to impact the market of bio-commodities and biomaterials within the next few years by providing chitin, calcium carbonate, and protein (Yan & Chen, 2015). A special emphasis is placed on chitin, (poly β-(1–4)-*N*-acetyl-D-glucosamine), which is the second most abundant natural polysaccharide after cellulose (Cho et al., 2000; Yadav et al., 2019; Zhu et al., 2016). Both chitin and its partially deacetylated homologue chitosan possess cyto-compatibility, biodegradability, analgesic, antimicrobial, antioxidant, anticholesterolemic and antitumoral activity (Aranaz et al., 2012). Thanks to these characteristics, these biopolymers have found applications in biomedicine, as food preservatives and as excipients in cosmetics, for antimicrobial packaging, and in biocatalysis (Bisht et al., 2021). Albeit present as a structural component of several terrestrial life forms such as insects, fungi and yeasts, chitin is mostly obtained from the exoskeleton of aquatic arthropods (Vázquez et al., 2013; Verlee et al., 2017; Yuan et al., 2020). Its total annual production is estimated around 2.8×10^{12} kg and 1.3×10^{12} kg from species of freshwater and marine ecosystems, respectively, the latter including prawns, crabs, shrimps, and lobsters (Cauchie, 2002).

Crustacean exoskeletons are composed of ca. 20–30 % chitin, 30–50 % minerals (mainly calcium carbonate), 30–40 % proteins and other substances including lipids (up to 14 %) and pigments (e.g., astaxanthin) (Peniche et al., 2008). Since chitin is insoluble in water (fortunately so for aquatic organisms) and in most organic solvents, its isolation is a laborious multistep process based either on chemical or on biological (microbial) methods (Kaur & Dhillon, 2013). Chemical pulping involves initial crushing and/or milling of the biomass, followed by deproteinization (DP), demineralization (DM), and decolouration to remove

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protein, inorganic components and pigments (astaxanthin, canthaxanthin, astacene, lutein and β-carotene), respectively (Tolaimate et al., 2003; Waśko et al., 2016). DM is commonly performed with dilute acids such as hydrochloric, nitric, sulphuric, acetic or formic, while DP is carried out using alkaline solutions for relatively long times at high temperatures (1–72 h at 65–100 °C); finally, decolouration involves solvent extraction with acetone, ethanol, ethyl acetate or their mixtures (Dahmane et al., 2014; Roberts, 1992). These different treatments imply several drawbacks. On one hand, they may induce potential adverse effects on the properties of extracted chitin: e.g., if acid/basic conditions for DM and DP are not strictly controlled, hydrolysis of the glycosidic bonds causes forming of oligomers (Percot et al., 2003), and the degree of acetylation (DA) and the crystallinity index (CI) can decrease (Li et al., 1997). Moreover, compared to biological methods, chemical procedures are aggressive for ecosystems, energy-intensive (Kaur & Dhillon, 2013), time consuming and generate large amounts of wastewater. For example, ~1 L (972 mL) of concentrated HCl (35 wt%) is required to demineralize 100 g of chitin from crab shell waste, generating 220 g (112 L at STP) of carbon dioxide in the atmosphere (Shamshina & Abidi, 2022). Nonetheless, chemical treatment of arthropod shells is still the preferred commercial route for the extraction of chitin.

In search of alternative solvents for chitin, other approaches were conceived by the combined application of microwave energy and highly solvating ionic liquids. For example, 1-ethyl-3-methylimidazolium acetate ([Emim][OAc]) proved effective not only to dissolve chitin, but also shrimp shells (Qin et al., 2010). Further investigations by the Rogers group highlighted that cheaper and more easily available ILs such as 2-hydroxyethylammonium acetate ($[NH_3(CH_2)_2OH][OAc]$), and hydroxylammonium acetate ($[NH_3OH][OAc]$) were effective for pulping of shrimp shells (Shamshina et al., 2016). Chitin was obtained in good yields (ca. 75–95 %) with around 70–80 % purity using 10 % loading of biomass in the IL. Among the stated drawbacks, the authors identified hydrolysis of the native chitin present in the shells due to the highly acidic nature of hydroxylammonium acetate that afforded lower MW chitin. Additional issues are posed by the toxicity and explosive nature of hydroxylamine, by the large amounts of ILs that in view of an economically viable process must be recovered and reused, and by the nature of the biomass source (shrimp shells are more easily pulped compared to harder crab shells) (Shamshina, 2019). We therefore initiated studies on the pulping of crustacean shells based on the seminal work by Rogers et al. with the aim of developing a more accessible, efficient and general pulping protocol applicable to a wider variety of crustaceans. Our pulping technique is based on cheap, safe and easily available ILs (Kalb, 2020), and targets spider crab shells (*Maja Squinado* (Pires et al., 2017)), one of the biggest and most abundant crabs, that in our view represents a benchmark waste biomass source for chitin extraction. Spider crab shells possess a higher chitin content and are more tenacious and resistant to processing than commonly described shrimp shells (Bastiaens et al., 2019). A reported procedure for the treatment of crab shells as a biowaste described the use of 1-allyl-3-methylimidazolium bromide ([AMIM][Br]) but due to the tenacity of the matrix the extracted chitin required further purification with aqueous citric acid to remove the excess calcium carbonate yielding chitin in only 13 % yield (Feng et al., 2020; Setoguchi et al., 2012).

In the present work, the pulping of *Maja Squinado* shells was carried out with ammonium acetate (AA) and ammonium formate (AF), two easy to synthesize and low-cost ILs which were prepared either *ex-ante* or by sequential addition of aqueous ammonia and acetic or formic acid to the biowaste (Fig. 1). The ILs contain both an alkaline anion and an acidic cation that contribute to the deproteinization and the demineralization of the biowaste in a single step.

Chitin was obtained in ca 17 wt% yield (representing a quantitative extraction yield calculated based on the chitin content in the dry biomass), and with a MW, crystallinity, and DA of 6.6×10^5 g/mol, 39–46 %, and 94 %, respectively. The pulping performance of AA and AF were compared to that of hydroxylammonium acetate (HA) which, to

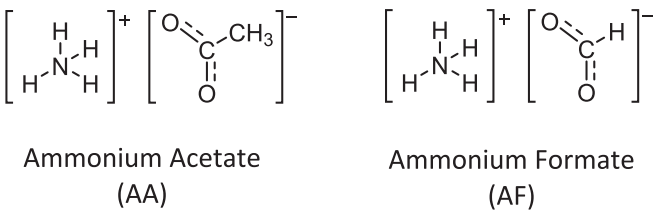

Fig. 1. Structure of the ILs used in this study.

date, is reported as the most effective IL for the pulping of shrimp shells (Kalb, 2020).

2. Results and discussion

2.1. Characterization of the starting feedstock

Spider crab shells were sourced as a waste of a local restaurant in Venice. The shells were initially ground, and the powder was analyzed by TGA and FT-IR. Results are reported in the SI section (Figs. S4 and S5). The thermogram displayed three main weight losses of ca. 6, 19 and 32 %, at around 100, 330 and 740 °C, associated with loss of water, decomposition of the organic fraction and decarboxylation of calcite, respectively (Kaya et al., 2014). The FT-IR spectrum exhibited bands consistent with O—H and N—H stretching vibrations and the presence of amide and carbonate groups. Overall, both TGA and FT-IR confirmed the presence of proteins, chitin, and a high mineral content, mainly calcite. Next, the chemical composition of the shells was determined: inorganics were measured by the Standard Test Method for Ash in Biomass (E1755–01, see Experimental section for details); while protein content was measured by difference between dry biomass and deproteinized biomass. The measured contents of ash and protein were 58 wt% and 21 wt% respectively, relatively to the weight of the starting dry biomass, thereby confirming that inorganic matter was the major component of crab shells. The percentage of chitin was estimated by difference, around 20 wt%. Finally, the content of calcium salts, mainly carbonate, was quantified by ICP-OES, resulting to be 52 wt% on dry basis. This result was consistent with the ash content analysis and proved that almost 90 % of ash residue in the treated biowaste was due to the presence of calcium salts. Lipids, carotenoids, and metal traces constituted only a minor fraction of the matrix. All these results (chemical composition and ICP analyses) were in good agreement with previous works on the characterization of spider crab shells of *Maja Squinado* (Pires et al., 2017).

2.2. Conventional stepwise extraction of chitin from spider crab shells

A sample of ground crab shells was subjected to a conventional stepwise procedure for the extraction of chitin with HCl and NaOH. This was adapted from a reported method (Sagheer et al., 2009), (see Experimental section for details). Chitin was obtained as a white solid in 17 % yield, and characterized by TGA, FT-IR and ^{1}H NMR. The characterization data were compared to those of a commercial sample of chitin. Results are reported in the SI section (Fig. S6-S8 and S11-S12). TGA showed two weight losses of 5 and 70 % at 100 and 334 °C, correlated to the release of water and to the decomposition/depolymerization of the material through deacetylation and cleavage of glycosidic linkages, respectively. A further progressive weight-loss at higher temperatures (415–670 °C) was related to the thermal destruction of the pyranose ring and the decomposition of the residual carbon containing fraction. The FT-IR spectrum of both commercial and conventionally extracted chitin (See SI Figs. S8 and S12) exhibited signals between 1700 and 1000 cm^{-1}, related to stretching of C═O in amide I group in the neighboring intrasheet chain, to hydrogen bonding between carbonyl and hydroxyl-methyl groups of two-chitin moieties in

the same biopolymer chain, to N—H bending (amide II) and to C—N stretching (amide III), respectively. Compared to the IR spectrum of the ground shells, the disappearance of the carbonate band (around 1400 cm^{-1}) and the sharp peak at 870 cm^{-1} typical of calcite confirmed the removal of the mineral portion (Pap et al., 2020).

The protein presence and DA of the conventionally extracted chitin were determined by ^{1}H NMR (see Fig. S11 and Experimental section for signals assignment). To overcome the difficulty posed by the poor solubility, a procedure was adapted from the literature (Einbu & Vårum, 2008) whereby a mixture of DCl (35 wt%) in D_2O was employed as NMR solvent. No resonances other than the ones attributed to chitin were observed, indicative of good purity. The ^{1}H NMR spectrum of chitin was however highly dependent on the time needed for dissolution of the sample. As an example, Fig. 2 shows the NMR spectra recorded at 10, 20 and 30 min after the dissolution of the polymer. Indeed, as shown in Fig. 2, the appearance and gradual increase of depolymerization characteristic signals became evident with extended treatment times.

In more detail, a comparison of the spectra indicated a change of the relative intensities of the different signals assigned to anomeric H-1 in the region between 4.5 and 5.5 ppm after 10, 20 and 30 min. This change indicated a decrease of H-1 present in the native polymeric chitin chain favoring the appearance of α-H-1 and β-H-1 typical of the terminal proton upon hydrolysis (depolymerization) of the glycosidic bonds due to the acidic conditions. With this in mind, all NMR samples were prepared under identical standardized conditions, specifically the spectra were recorded in DCl/D_2O mixture, after 30 min from dissolution. Under these conditions, depolymerization certainly occurs in the NMR tube in the presence of DCl to yield a mixture of oligomers and glucosamine monomer, implying that the solution does not contain native chitin. Nonetheless, the rate constant for hydrolytic depolymerization is much faster than deacetylation (Einbu & Vårum, 2008) allowing to use ^{1}H NMR to measure the degree of acetylation (DA) of chitin. DA was determined based on the resonances of the characteristic protons and according to eqs. 3 and 4 described in the experimental section. DA of 92 % was determined for both the extracted and the commercial chitin, respectively.

This preliminary investigation led us to conclude that the conventional treatment of the waste spider crab shells allowed to extract chitin which, based on characterization by TGA, FT-IR, and NMR, showed properties comparable to those of a commercial chitin specimen.

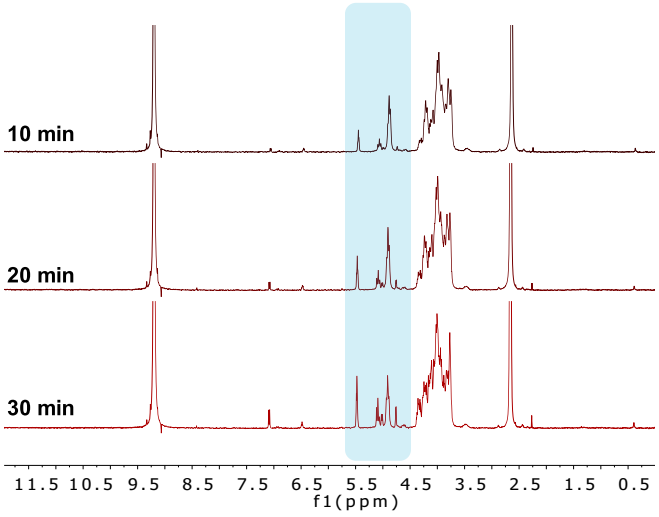

Fig. 2. Comparative ^{1}H NMR spectra of commercial chitin in DCl 35 % at 50 °C, after 10, 20 and 30 min, respectively.

2.3. Pulping strategies based on ILs for chitin isolation

The complexity of biomass and especially biowaste processing is such that any change on the feedstock requires rethinking the treatment conditions and the implementation of novel protocols. Indeed, the lack of broad-based conversion technology is still a major challenge of modern biorefineries (Bozell & Petersen, 2010), and the IL-assisted extraction of chitin from crustacean shells is not an exception. Most, if not all, of the available studies on the pulping of arthropods shells with ionic liquids for the extraction of chitin have been referred to shrimp shells as the starting feedstock. The seminal work by Shamshina et al. (2016) pioneered this protocol and demonstrated the efficacy of hydroxylammonium acetate as an extractant medium, and also explored the transition of the IL-based extraction from academia to an industrial scale (Kalb, 2020). As anticipated in the introduction, spider crab shells are rich in chitin but more tenacious and resistant to processing than shrimp shells. Therefore, in view of developing a general protocol we focused on spider crab shells, with the aim of minimizing the environmental impact of chitin isolation by choosing greener and cheaper ILs able to operate in a single step and with reduced extraction volumes. The ILs selected for this study were commercially available ammonium acetate (AA) and ammonium formate (AF). These are protic ammonium salts derived from a weak acid and a weak base, which have found applications in energy storage and in the treatment of lignocellulosic biomass (Greaves & Drummond, 2008; Schiffer et al., 2022). The behavior of both, AA and AF, was compared to that of hydroxylammonium acetate (HA) which was reported the best IL for the pulping of shrimp shells. HA was synthesized according to a method reported elsewhere (see Experimental section and SI, Fig. S1-S3) (Shamshina et al., 2016).

The powdered crab shells (PCS) were firstly treated by three different approaches (1,2,3) which are summarized in Fig. 3: 1) neat AA, AF, or HA prepared ex-ante (or purchased) was directly mixed with the PCS and heated at a temperature of 130 °C, high enough to melt the onium salt (left); 2) acetic acid or formic acid was mixed with PCS, then aq. ammonia or hydroxylamine was added dropwise to generate in-situ the corresponding onium salts (middle) and the resulting suspension was heated at 100 °C under stirring; 3) the in-situ formed onium salts were obtained by reversing the addition order of point 2) [1) base→2) acid (right)].

The PCS was always 10 wt% of the total mass of suspension.[24] Heating was carried out for 2 h in all tests. Thereafter, the suspension was cooled, added with water, and filtered. The residual white solid was further rinsed with water, dried under vacuum, and analyzed by TGA, ICP-OES, NMR and FT-IR (Further details are in the experimental section).

All characterization data confirmed the obtainment of chitinous material. However, the use of different onium salts and different procedures (1, 2, 3) resulted in different yields ranging from 16 to 30 %. Results are reported in Table 1.

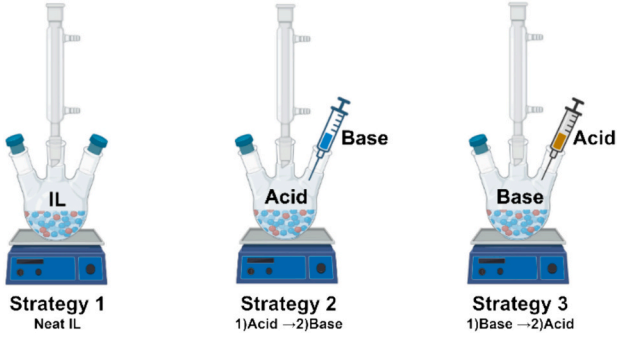

Fig. 3. Schematic representation of the pulping strategies employed for chitin isolation.

Table 1
Experimental conditions and outcome of the pulping strategies (1–3) for chitin isolation.

Entry	IL	Strategy	T (°C)	Extraction Yield (%)	$CaCO_3$ (wt%)[a]		DA (%) by NMR[c]	
					By TGA	by ICP-OES[b]	Method 1	Method 2
1	AA	1	130	16	ND	4	99	99
2		2	100	30	14	33	88	93
3		3	100	27	9	19	97	97
4	AF	1	130	20	6	11	94	95
5		2	100	17	ND	1	97	97
6		3	100	18	ND	1	94	95
7	HA	1	130	29	10	10	–	–
8		2	100	20	7	7	96	97
9		3	100	21	6	6	93	94

The pulping of PCS was carried out for 2 h in all cases.
[a] Amount of $CaCO_3$ (wt%) in the solid isolated after the pulping.
[b] Calcium determined by ICP-OES was considered in form of carbonate salt. ND: non detected.
[c] The degree of acetylation (DA) was determined by NMR according to two methods based on Eq. 3 and Eq. 4, respectively, reported in the experimental section.

TGA and ICP-OES analyses indicated in most cases that the extracts contained a variable residual amount of $CaCO_3$ (Table 1, entries 2–4, 7–9) from 9 to 33 % of the weight of the samples, indicating the incomplete DM of the starting bio-waste. In particular, TGA profiles showed a weight loss between 700 and 800 °C, due to the release of CO_2 from calcium carbonate (Figs. S14-S16, S19–21), while, upon digestion of the extracts under acid conditions, Ca^{2+} was detected by ICP-OES and quantified in the resulting solutions (details are in the experimental). These preliminary results explained why in some cases during the AA- and the HA-assisted pulping, regardless of the temperature, the extraction yield was apparently higher (27–30 %: entries 2, 3, 7) than the theoretical chitin content in crab shells (17 %).

The most efficient demineralization was achieved by procedures 2 and 3, through the AF-assisted pulping of PCS at 100 °C: in this case, not only the yield (17–18 %) was consistent with the expected amount of chitin in the starting biowaste, but both TGA and ICP-OES confirmed a negligible content of residual mineral ($\leq$ 1 wt%; entries 5–6). A representative TGA curve of chitin sample of entry 5 is displayed in Fig. 4A. The profile was comparable to that of commercial chitin (Fig. S6). The more efficient removal of minerals by AF was demonstrated also by the FT-IR spectrum of Fig. 4B. Under the same pulping conditions, the use of HA gave higher yields (20–21 %) with, however, higher amounts of $CaCO_3$ (6–7 wt%, entries 8–9, and Figs. S20–21). A representative FT-IR spectrum of chitin pulped with HA at 100 °C is reported in Fig. S22).

This evidence led us to conclude that for the extraction of chitin: i) the direct suspension of PCS into ILs (procedure 1) was less efficient than sequential additions of acid(s) and base(s) via procedures 2 and 3, respectively. A possible explanation for this behavior was that the higher viscosity of neat ILs, compared to that of the in-situ synthesized ones where water is present, hindered the diffusion and efficient interaction of the pulping agent within the chitin chains, thereby resulting in an incomplete DM; ii) apparently, the order of addition (acid first then base or vice versa), was almost uninfluential on the yield of the extracts and their residual mineral content (compare entries 2–3, 5–6, and 8–9); iii) acetic acid, responsible for the DM in AA and HA, due to its lower acidity in comparison with formic acid, was less effective for the complete removal of minerals at 100 °C. Instead, by using in-situ formed AF, complete DM could be achieved.

^{1}H NMR analyses of the samples of Table 1 were carried out under the same conditions of Fig. 2 (30 min). A representative spectrum is reported in Fig. 5 (bottom) for chitin obtained by the AF-assisted pulping (entry 5, Table 1). For comparison, the spectrum of commercial chitin is also reported (top). Both spectra showed all the characteristic signals of the glucosamine structural units; though, the spectrum of the pulped chitin displayed an additional small signal between 1.0 and 1.5 ppm (highlighted box on the right). This was attributed to the resonance of protons attributed to methyl groups of the residual proteins present in the treated crab shells. To verify this hypothesis, the pulped chitin sample was subjected to chemical deproteinization (DP) with aq. NaOH, according to the described conditions (Sagheer et al., 2009). After this treatment, the NMR analysis confirmed that the high-field signals at 1–1.5 ppm had disappeared, thereby substantiating the removal of proteins and the proposed assumption. Ton confirm removal, residual proteins were also measured by UV–Vis spectroscopy (see Fig. SX). NMR spectra and UV–Vis spectra of pulped chitin before and after DP are reported in the SI section (Fig. S25).

Further NMR spectra of the samples of Table 1 are reported in the SI (Figs. S25-S27). These spectra and the ones of Fig. 5, allowed to calculate the degree of acetylation (DA) of pulped chitin. Pulping with AA,

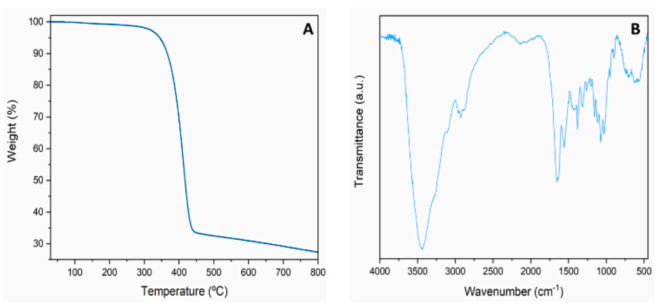

Fig. 4. A: TGA curve and B: FT-IR spectra of chitin pulped with AF at 100 °C.

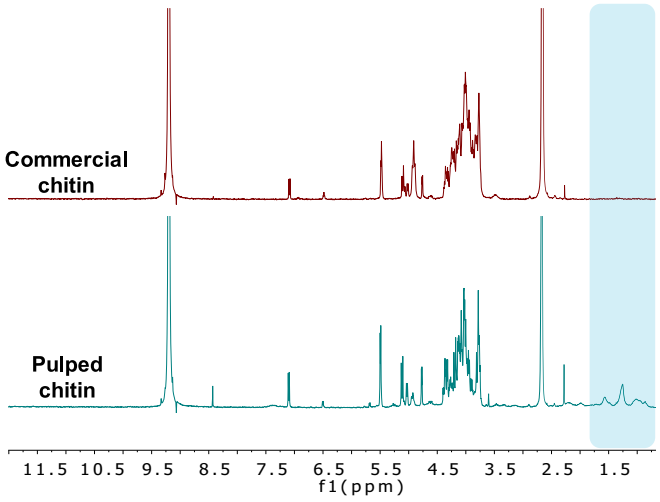

Fig. 5. Comparative ^{1}H NMR spectra of a representative sample of chitin pulped with AF (bottom, green) and of commercial chitin (top, red). (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article.)

AF, and HA yielded DA values higher than 94 %, regardless of the strategy 1, 2 or 3 used (Table 1). An exception was the AA-assisted extraction with procedure 2 that gave DA of 88–93 % (entry 2, Table 1). Moreover, when neat HA was used (entry 7), the determination of the DA was hindered by the overlapping of H-2 signals of internal de-*N*-acetylated units with impurities possibly still present in the sample.

Since AF was found to give the best performance, further analyses of the chitin samples obtained using this IL were accomplished, including XRD (Fig. S9-S10, S24, SI), which gave insight on the crystallinity of the pulped samples (Hong et al., 2018). The samples obtained by using AF as a solid salt or by sequential addition of formic acid followed by ammonia displayed comparable CI (ca. 39 % in both cases), while by using the strategy 3 (base first, then acid), a slightly higher CI value of 46 % was determined.

2.4. Parametric analysis of the pulping procedure

Having established AF as the best IL for chitin isolation, but that residual proteins were still present after the pulping procedure, a parametric analysis of process conditions was accomplished, considering the influence of biomass wt% loading, temperature and time. For this purpose, initial experiments were carried out following strategy 2, the less- energy intensive (100 °C), selected as the preferred option from the preliminary experiments. We studied the effect of biomass loading and temperature, by increasing the crab shell amount from 5 wt% up to 20 wt% and the pulping time up to 24 h, at 100 °C. FT-IR and NMR analyses were carried out for the pulped chitin to evaluate the efficacy of DM and DP of the crab shells. Results are reported in Figs. S23, S26-S27.

Apparently, neither the biomass wt% loading nor the prolonged pulping time affected the characteristics of the final extracts. Experiments confirmed that for all samples, the extraction yield was in the expected range (17–18 %), the mineral content was negligible (carbonate decomposition was never detected by TGA), and the DA (≥94 %) was comparable to that reported in Table 1. Though, the presence of residual proteins was observed in all cases, indicative of incomplete DP.

Considering such results and in view of further scalable applications, 20 wt% biomass loading was selected for further tests. These were designed according to procedure 2 by increasing the pulping temperature up to 150 °C and evaluating the DP progress in time. Experiments were run for 2, 3 and 4 h. Fig. 6 shows the results. Complete DP was achieved after 4 h, as indicated by the disappearance of the signal between 1.0 and 1.5 ppm.

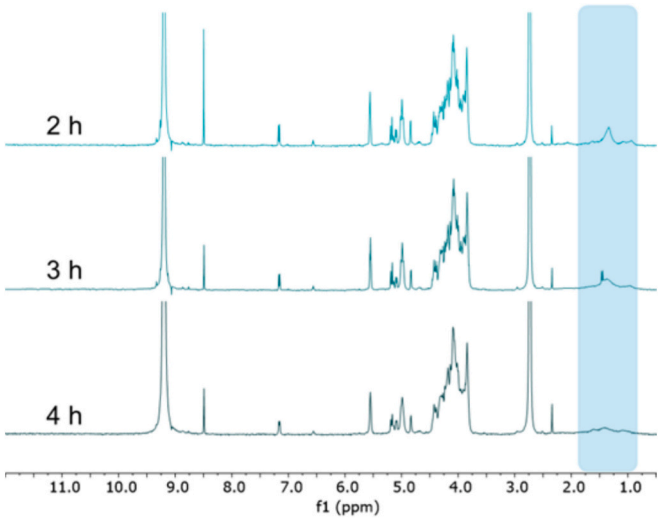

Fig. 6. Comparative ^{1}H NMR spectra of the influence of pulping time on the chitin purity and DP efficiency. Pulping conditions: 150 °C, 20 wt% biomass loading.

A scalability test was then carried out under the conditions of Fig. 7 (150 °C for 4 h) by increasing the amount of the treated biomass by a factor of 5 (2.5 g of PCS). Remarkably, no mineral content was present in the pulped chitin, but residual proteins were detected by NMR (Fig. S28) indicating that a further process optimization was needed at higher scales to increase the efficiency of DP. This (optimization) study was beyond the scope of the paper; the results, however, paved the way for the engineering of a new protocol for the sustainable IL-assisted extraction of chitin from crab shells.

2.5. Further characterization of the pulped chitin

2.5.1. Molecular weight

The MW distribution of the pulped chitin was evaluated for 6 different samples obtained by the procedure 2 carried out at different temperatures (100 and 150 °C), times (2–24 h), and biomass loading. Each sample was dissolved in a 5 % LiCl/NMP (*N*-methyl-2-pyrrolidone) solution (Chen et al., 2014), and analyzed by GPC, using polystyrene as internal standard (details are in the experimental section). Molecular weight and polydispersity are reported in Table 2 while GPC curves are reported in the SI (Fig. S30-S36).

Compared to commercial chitin (entry 7), samples extracted at 100 °C for 24 h exhibited a nearly double M_W regardless of the IL-substrate ratio (entries 1–3) indicative of reduced hydrolysis under these conditions. Polydispersity is equally high for both commercial and pulped chitin, though in the first case it is caused by the broad but symmetrical molecular weight distribution and for the later an additional high molecular weight component appears. This can be attributed to the presence of protein residues still anchored to the chitin fibres extracted through the pulping process at 100 °C (as can be also seen in Fig. S27), mainly due to the lower aggressiveness of these ionic liquids compared to the conventionally used acids and bases bases (Hong et al., 2018; Qin et al., 2010). Pulping at 150 °C for 2–4 h resulted in both the M_W of the extracts decreased to approach the value of commercial chitin and complete DP indicated by a symmetrical distribution with polydispersity (PD) narrower than that for the commercial chitin (entries 4–6). This would allow to expect improved strength and toughness of the IL-pulped chitin compared to acid-assisted one while still remaining its processability. Overall, the higher temperature contributed to a complete DP of the samples accompanied by a degree of depolymerization similar to the one achieved through strong acid/base, yielding nonetheless chitin with good M_W and a narrower PD, further supporting the reliability, the reproducibility, and the efficacy of the pulping process in comparison with conventional methods.

An estimation of the residual amount of proteins was carried out by Near UV Absorbance (280 nm). The absorption of radiation in the near UV by proteins depends on their Tyr and Trp content (and to a lesser extent on the amount of Phe and disulfide bonds). Two representative samples, namely the chitin pulped at 100 °C for 24 h and the one pulped at 150 °C for 4 h, were analyzed in detail. The measurements were repeated four times to ensure reproducibility. The graph has been included in the supporting information file (Fig. S37). It was observed that under the optimized conditions, a clear reduction (by four times) of the protein content was achieved.

2.5.2. SEM analyses

The morphological characterization of the chitin extracted under the conditions of Fig. 7 (150 °C, 4 h) was accomplished by SEM. Fig. 7 compares SEM images of ground crab shell (Fig. 7A and B), commercial chitin (Fig. 7C and D), chemically extracted chitin (Fig. 7E and F), and the pulped chitin (Fig. 7G and H). The differences between the samples are significant: the ground crab shells showed a compact texture sealed with proteins and carbonate, while in the commercial chitin from shrimp shells and in the conventionally extracted chitin from ground crab shell DP and DM revealed a porous network of chitin fibres, with much less or no granules on the surface. Finally, the IL-pulped chitin

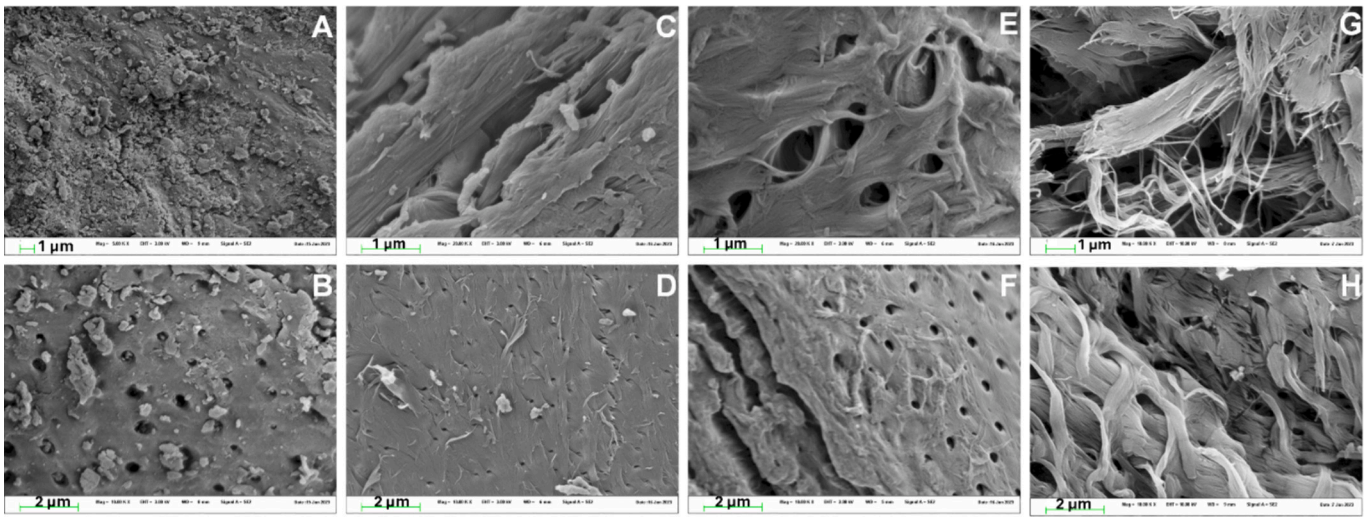

Fig. 7. SEM images of A-B: Ground crab shell; C-D: Commercial chitin; *E*-F: Chemically extracted chitin; G-H: Pulped chitin (obtained in this work under optimized conditions). (Magnifications: A: 5.00 K; B, D, G, F, H: 10.00 K; C,E: 20.00 K).

Table 2
MW and PD data of representative pulped chitin samples.

Entry	t (h)	T (°C)	wt% loading[a]	M_W (g/mol)*10^5	PD
1	24	100	5	11.9	2.2
2	24	100	10	11.4	2.5
3	24	100	20	11.4	2.3
4	2	150	20	6.7	2.0
5	3	150	20	4.3	1.7
6	4	150	20	5.1	1.9
7	Commercial chitin			5.9	2.3

[a] wt% of biowaste (PCS) compared to the total mass of the suspension (IL + biomass) treated by procedure 2.

displayed a highly fibrous morphology with loose and separated fibres confirming effective pulping but also unique effect of the IL-assisted extraction that allowed to avoid fibre hornification. The IL-treatment was likely less aggressive than other methods and helped to preserve the native morphology of the biopolymer and also secured its better processability toward materials. Such results are promising also in view of further functionalisation of the extracts (for example, via deacetylation to obtain chitosan), that could be favoured by a better diffusion of the reactants inside the fibrous network (Cárdenas et al., 2004; Chandran et al., 2016).

2.6. Insights into the effect of formic acid on the chitin arrangement

Pulping tests demonstrated that AF was the most efficient salt for the extraction of chitin from crab shells. However, ^{1}H NMR spectra of the samples obtained by the AF treatment, particularly when formic acid was used via procedures 2 and 3, displayed an additional signal at 8.4 ppm (blue box on the top of Fig. S29, and Fig. S38) which was consistent with the resonance typically associated to the singlet peak of an aldehydic moiety (-COH) (Hudson et al., 2020). Interestingly, even after neutralization by a basic solution (NaOH, 1 M), the low-field signal was still observed, suggesting that possibly an ammonium formate was likely entrapped within the network of polysaccharide chains. Another possibility is the interaction between the free $–NH_2$ groups or secondary hydroxyl groups of chitin with formic acid formed by partial degradation of AF at $T > 120$ °C (Jaekel et al., 2021). To probe this hypothesis, the pulping of PCS was performed via the procedure 2 (150 °C, 4 h), by replacing HCOOH with DCOOH. The NMR spectrum of the extracted chitin did not show any signal at 8.4 ppm (Fig. S38, blue box on the bottom). This confirmed our assumption and suggested the occurrence of a strong interaction between the pulped chitin and formic acid.

2.6.1. Sustainability assessment of the IL-assisted pulping

The sustainability of the here proposed pulping method was evaluated by using the unified metrics toolkit proposed by Clark and coworkers within the CHEM21 project (McElroy et al., 2015). Although originally designed to compare the greenness of chemical reactions, the metrics used by CHEM 21 provided a benchmark to monitor the green credentials of the extraction protocol at least within the limits of the zero/first pass analysis that characterises the structure of the metrics toolkit. Particularly, the parameters that were taken into consideration, included the extraction yield (Y), the process mass intensity (PMI), the impact of the workup techniques, the energy use (E), and H-statements to identify chemicals (reagents and solvents) of concern (health and safety). This analysis compared the pulping carried out by ammonium formate using the procedure 2 (AF-P2) to the conventional extraction process (CEP). The results are reported in Table 3.

The output of the CHEM21 metric toolkit (CMT) are green, amber or red indicators that mark acceptability of a given variable: green ‘preferred’, amber ‘acceptable-some issues’ and red ‘undesirable’. The CEP and AF-P2 protocols provided a comparable 85 % yield of chitin based on the maximum amount of chitin (20 wt%) in the dry biomass used as a feedstock. Such a yield (< 90 %) ranked both procedures with an amber flag.

The evaluation of the workup techniques and of the H-statements of the reagents did not discriminate between the two methods of chitin extraction: both ranked one amber and two green flags, respectively. However, the AF-P2 pulping was a single step protocol which was remarkably more advantageous in terms of step economy and process intensification compared to the two-step CEP (McElroy et al., 2015). While this ranking effectively mitigated the environmental burden of the pulping, nonetheless it was partly offset by the higher energy demand of the method that, especially when carried out at 150 °C (E: red flag), was out of the acceptable standard temperatures defined by the CHEM 21 toolkit.

The most striking difference between the two extraction procedures emerged from the comparison of one of the most important metrics that measures all mass-based inputs – process mass intensity – defined as the ratio between the total mass used in a process and the mass of the desired product. Thanks to a more efficient and reduced use of reagents in the AF-P2 protocol, the corresponding PMI was >8-fold lower than that of the CEP (details on the calculation of PMI are in the SI section, Table S2). This result contributed the competitiveness of the AF-assisted

Table 3
Evaluation of chitin extraction processes from ground crab shells using CHEM21 metrics toolkit.

Entry	Extraction procedure		Y (%)[b]	PMI (g/g)[c]	Work-up		E (T/°C)	Health and Safety
1	Chemical (CEP)	Step 1: DM[d]	~85	241	Neutralisation[f]	Centrif[g]	25	HCl: H290, H314, H318, H335
		Step 2: DP[e]			Neutralisation	Centrif	70	NaOH: H290, H314, H318, H402
2	Pulping AF-P2 [a]	Single step	~85	29	Aq. Washing[f]	Centrif	100 150	AF: (Jacobs et al., 2023)

[a]Pulping of chitin assisted by ammonium formate carried out using the procedure 2 described in this work. [b] Yield of extracted chitin based on the maximum amount of chitin (20 wt%) on the dry biomass used as a feedstock. [c] Process Mass Intensity. [d] DM: demineralization. [e]DP: deproteinization. [f] Before centrifugation, neutralization or washing with fresh water of the extracted chitin were necessary. These operations were considered as equivalent to a quenching into water (McElroy et al., 2015) [g] Centrifugation.

pulping compared to CEP and pointed the way for its future optimization.

Finally, a rough techno-economic analysis based on the standard costs of energy (in Italy) and research-grade reagents supplied by Merck and used in this work, was carried out to estimate the price of chitin pulped from crab shells. This worst-case-scenario estimate indicates to a production cost of this chitin at 205 €/Kg. Notably, this sample exhibited a molecular weight of $5.1 \bullet 10^5$ g/mol and a DA >94 % that placed it on par with a Merck commercial sample (MW = $5.9 \bullet 10^5$ g/mol and DA = 94 %), with a price point of 311 €/Kg. Details of this analysis are reported on Table S3.

The real overall cost of the pulping would be much lower by optimizing both the utilization of reagents and solvents through their sourcing via more cost-effective suppliers for bulk orders and the scale-up of the protocol (see Table S3). Additionally, enhancing the market acceptance of IL-assisted pulping technology would further contribute to cost reduction.

3. Conclusions

In conclusion, a sustainable and cost-effective one-pot process for chitin pulping from crab shells was developed, using easily available, affordable and non-hazardous ionic liquids, following three different strategies (as a solid salt or by in-situ preparation). In particular, in-situ prepared ammonium formate (AF) exhibited the best pulping performance being the most promising candidate and allowing quantitative isolation (17–18 % yields) of chitin with high purity and a high degree of acetylation (DA > 94 %). AF resulted to be highly efficient in mineral removal leading to complete dissolution of all inorganic salts (confirmed via TGA, ICP-OES and FT-IR analyses) present in the starting material with a 2 h treatment. A complete parametric analysis was carried out to improve the DP step, achieving the best results at 150 °C for 4 h with complete protein removal. Noticeably, chitin with a high DA and a MW was obtained, paving the way toward an innovative, economically effective and greener solution for chitin preparation, replacing the current hazardous processes with high environmental impact.

4. Experimental section

All reagents and solvents were used without further purification. Commercial chitin from shrimp shells, NaOH, HCl, NH_2OH aqueous solution 50 wt%, acetic acid, NH_3 solution 30–33 wt%, formic acid, ammonium formate salt, ammonium acetate salt and KBr were ACS grade and were all purchased from Sigma-Aldrich. Spider crab shells were obtained from a local restaurant in Venice, already washed with a detergent solution. Shells were ground and then sieved into three different meshes (20, 60 and ≥ 150). Powders of spider crab shells were weighed and stored in glass bottles at ambient temperature before usage.

4.1. Crab shells composition

Ash content was determined using the Standard Test Method for Ash in Biomass E1755-01. The ash content was calculated with Eq. 1 where %ash is the mass percent of ash, m_{ash} the final mass of ash and container (g), m_{cont} the mass of container (g), mar the initial mass of biomass sample and container (g).

$$\%ash = \frac{m_{ash} - m_{cont}}{m_{ar} - m_{cont}} \times 100 \quad (1)$$

Protein content was determined by chemical deproteinization of crab shells (see SI). Protein percentage with respect to crude shell dry weight was then extrapolated using Eq. 2 where $m_{residue}$ is the total dry mass of residue free of proteins and $m_{biomass}$ refers to initial mass of dry shells.

$$\%proteins = 100 - \left(\frac{m_{residue}}{m_{biomass}} \times 100\right) \quad (2)$$

Chitin percentage was estimated by employing the conventional chemical extraction method (see SI).

4.2. Characterization of synthetised ILs

^{1}H NMR and ^{13}C NMR. ^{1}H NMR and ^{13}C NMR spectra were obtained using a Bruker NMR spectrometer Ascend 400 (AV400), operating at 400 MHz for ^{1}H nuclei and 100 MHz for ^{13}C at ambient temperature (298 K).

4.3. Characterization of extracted chitin

The samples for FT-IR (Fourier-transform infrared spectroscopy) analysis were prepared by grinding the dry blended powders with powdered KBr, (ratio of 1:100 Sample: KBr) and then compressed to form pellets of 5 mm-diameter using a hydraulic press at a pressure of 10 tons for one minute. FT-IR spectra were recorded on a Perkin-Elmer Spectrum One FT-IR spectrometer. All spectra were recorded in the middle infrared (4000 cm^{-1} to 450 cm^{-1}) with a resolution of 4 cm^{-1} in the absorbance mode for 16 scans at room temperature. ^{1}H NMR analysis was carried out by dissolving chitin samples in a deuterium chloride solution (DCl 35 w.t % in D_2O) with vigorous stirring for 30 min at 50 °C. ^{1}H NMR spectra were obtained using a Bruker UltraShield 300 operating at 300 MHz at ambient temperature (298 K) for 64 scans with a spectral window of 20 ppm.

DA (degree of acetylation) was calculated from the NMR spectra using Eqs. 3 (based on the integrals of H-1 and H-2 signals) (Einbu & Vårum, 2008) and 4 (based on the area of the H-2 of the glucosamine (GlcN) units and of the acetyl protons of *N*-acetyl glucosamine (GlcNAc)

units) (Daraghmeh et al., 2011). Minimal deacetylation, which could have caused an increase in the signals related to acetic acid and the H-2 proton of de-*N*-acetylated chitin, occurred during the dissolution of all samples in the deuterated solvent.

$$DA\% = \frac{\left(I_{\alpha H1A} + I_{\beta H1D+H1D} + I_{H1A}\right) - I_{H2D}}{I_{\alpha H1A} + I_{\beta H1D+H1D} + I_{H1A}} 100 \quad (3)$$

$$DA\% = \frac{\frac{1}{3}A_{CH_3}}{\frac{1}{3}A_{CH_3} + A_{GlcN}} 100 \quad (4)$$

Chitin signals- ^{1}H NMR (300 MHz, DCl 35 % in D20, 298 K) δ (ppm): 6.94 (d, H-1*), 5.33 (br, α-H1A), 4.95–4.98 (br, β-H1A + H1D), 4.87–4.88 (d, H-2*), 4.77 (br, H1A), 4.61 (d, H-3*), 3.5–4.3 (m, H2/H6), 2.53 (s, H-Acetyl), 2.13 (s, H-acetic acid). Signals denoted with an asterisk refers to glucofuranosyl oxazolinium ion existing in equilibrium with GlcNAc in concentrated DCl.

The thermogravimetric (TGA) analyses were carried out in a TA instruments SDT 2960. About 5 mg of all samples were placed in aluminium crucible and heated from ambient temperature to 800 °C with a heating rate of 10 °C/min under a nitrogen atmosphere (flow rate 20 mL/min). Inductive Coupled Plasma-Optical Emission Spectroscopy (ICP-OES) measurements were performed on a Perkin Elmer ICP-OES 5300 DV. All chitin samples were digested in nitric acid (3 mL) and hydrochloric acid (2 mL), dilute to 10 mL of final volume, and heated at 150 °C for 20 min to ensure complete dissolution. X-Ray Diffractometry (XRD) measurements were performed employing a Philips diffractometer with a PW 1319 goniometer with Bragg-Brentano geometry, equipped with a focusing graphite monochromator and a proportional counter with a pulse-height discriminator. Nickel-filtered CuKα radiation and a step-by-step technique were employed (steps of 0.05° in 2θ), with a collection time of 30 s per step. The background signal was subtracted and the diffractogram corrected. Crystalline and amorphous domain areas were properly deconvoluted and separated to provide CI with good accuracies (Ioelovich, 2014). The actual degree of crystallinity (CI) of the sample was calculated, as follows (Eq. 5):

$$CI = \int I_{cr} d\theta \Big/ \int I_0 d\theta = F_{cr} \Big/ (F_{cr} + F_{am}) \quad (5)$$

where I_0 is total intensity of the corrected diffractogram after subtraction of the parasitic background; I_{cr} is intensity of the crystalline scattering; F_{cr} is area of the crystalline scattering; F_{am} is area of the amorphous scattering.

Molecular weights of the commercial and pulped chitin were determined by Gel Permeation Chromatography (GPC). For each analysis, 5 mg of chitin was solubilized in 5 % LiCl/NMP (sample concentration: 1 mg/mL) by stirring at room temperature until complete dissolution. The solution was then filtered through a 0.45 μm syringe filter. GPC analyses were conducted with a GPC-Agilent/PSS 1260 Iso Pump instrument by using a PSS-gram-100 and a PSS-gram-1000 column in series, after a PSS-gram-precolumn. The sample was eluted with 5 % LiCl-NMP (flow rate, 0.5 mL/min, injection volume: 100 μl, temperature, 70 °C, measuring time, 60 min) and PSS SECcurity-UV-300 nm-1260 VWD and PSS SECcurity-RI-1260 RID were used as detectors. Calibration curves of log (Da) from retention time were built by consequently measuring a sequence of PS (Polystyrene) internal standards with molecular weight ranging from 1306 to 2,520,000 Da.

4.4. Synthesis of hydroxylammonium acetate

Hydroxylammonium acetate was prepared according to a reported procedure (Shamshina et al., 2016). Hydroxylamine solution 50 wt% (26.42 g, 0.4 mol) was stirred at 0 °C in a 250 mL round-bottomed flask while acetic acid (26.4 g, 0.44 mol) was added dropwise over 3 h. The solution was then allowed to reach room temperature overnight under continuous stirring. The mixture was then gently purged with air and heated at 40 °C to concentrate the solution. The precipitate was washed with methanol (100 mL) and diethyl ether (100 mL) to remove residual acetic acid and dried in oven under reduced pressure (15 mbar) for 2 h. The final product was obtained as a white solid (11.9 g, yield 32 %) and was characterized by NMR spectroscopy (1H and 13C in DMSO-d_6 or D_2O). Characterization data are reported in SI.

4.5. Conventional stepwise extraction protocol

In a typical procedure, a powdered sample (1 g) of spider crab shells was suspended in aqueous HCl (0.5 M; 40 mL/g of waste) and stirred at room temperature for 15 min. The residual solid was centrifuged (6000 rpm for 10 min), water was added (5 × 50 mL) to neutral pH, and the resulting solid was dried in oven under reduced pressure (15 mbar) overnight. The demineralized powder was then suspended in aqueous NaOH (1 M; 20 mL/g of solid), at 70 °C for 3 h under continuous stirring. The solid product was centrifuged (6000 rpm for 10 min), washed with water (5 × 50 mL) until neutral pH, and dried in oven under reduced pressure (15 mbar) overnight. The final product was obtained as a white solid (0.169 g, yield 17 %).

4.6. Pulping of crab shells using IL as solid salt

Ground crab shells (0.5 g) were mixed with solid IL (4.5 g) in a round-bottom flask equipped with a condenser. The suspension was kept under magnetic stirring for 2 h at the chosen temperature (145 °C for AA, 130 °C for AF and 100 °C for HA). After complete melting of the IL, a vigorous bubbling/foaming could be observed. A yellowish solid precipitate after 2 h, while impurities were confined in the aqueous phase. The mixture was diluted with 50 mL of DI water and centrifuged. The solid was washed with water (5 × 50 mL) until neutral pH was reached and oven-dried under reduced pressure (15 mbar) overnight. The final product was obtained as a yellow-white solid.

4.7. Pulping of crab shells using IL prepared in-situ at 100 °C

0.5 g of ground crab shells were put in a two-necked round-bottom flask equipped with a condenser. Acetic acid or formic acid (1.8 mL and 1.5 mL, respectively) was added, followed by dropwise addition of aqueous basic solution (ammonia or hydroxylamine). The solution was kept under stirring for 2 h at 100 °C. Purification and isolation was performed as described in the pulping with solid salt. The same procedure was repeated by changing the addition order of reactants (first the base, then the acid). Used quantities are reported in Table S1.

4.8. Pulping of Crab shells using IL prepared in situ at T > 100 °C

0.5 g of ground crab shells were put in a two-necked round-bottom flask equipped with a condenser. The acidic function (acetic or formic acid) was added, followed by dropwise addition of aqueous ammonia solution over several minutes. The solution was kept under magnetic stirring for ca. 0.5 h at ambient temperature until foaming and bubbling occurred. The mixture was then placed in a stainless-steel autoclave equipped with a Teflon reactor and kept under magnetic stirring for 2 h at the desired temperature (145 °C with AA and 130 °C for AF). The autoclave was then allowed to cool to room temperature. Purification and isolation were performed as described in the pulping with solid salt. The same procedure was repeated by changing the addition order of reactants (first the base, then the acid). In all cases, a 10 wt% loading of biomass was used with respect to total mass of solution (IL and biomass). Used quantities are reported in Table S1.

4.9. Protein estimation by Near UV Absorbance (280 nm)

20 mL of 1.25 M NaOH solution was added to 50 mg of two representative dried chitin samples, and the mixtures were heated at 100 °C

for 2 h. After cooling to room temperature, the mixture was centrifuged to recover several aliquots of the supernatant, which were then stored in the refrigerator (~4 °C) for further protein analyses. The absorbance of the samples at 280 nm was measured using the Eppendorf BioSpectrometer® basic.

CRediT authorship contribution statement

Carlotta Campalani: Conceptualization. **Ilaria Bertuol:** Investigation, Conceptualization. **Chiara Bersani:** Data curation. **Roberto Calmanti:** Validation, Project administration, Data curation. **Svitlana Filonenko:** Investigation. **Daily Rodríguez-Padrón:** Investigation, Data curation. **Maurizio Selva:** Supervision, Methodology, Conceptualization. **Alvise Perosa:** Supervision, Resources, Conceptualization.

Declaration of competing interest

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

Data availability

Data will be made available on request.

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Appendix A. Supplementary data

Supplementary data to this article can be found online at https://doi.org/10.1016/j.carbpol.2024.122565.

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