Deep eutectic solvents for the extraction of β-chitin from Loligo vulgaris squid pens: a sustainable way to valorize fishery by-products

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Deep eutectic solvents for the extraction of β-chitin from *Loligo vulgaris* squid pens: a sustainable way to valorize fishery by-products

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Abstract

β-chitin, a promising biopolymer for the production of chitosan and biomaterials, is sourced from fishery by-products. Herein, β-chitin has been extracted using Deep Eutectic Solvents (DES) for the first time, only the α-polymorph having been extracted using these solvents until now. Six acid, neutral, and alkaline DES systems were trialed, with only the alkaline DES systems, in particular potassium carbonate: glycerol (KGLY) at 100 or 120 °C for 2 or 3 h, resulting in high-purity β-chitin. The ensuing β-chitin was characterized in terms of chemical and physical structure, morphology, crystallinity, and thermal properties. Under the best extraction conditions, using the DES KGLY system, the β-chitin samples were highly pure, presenting an acetylation degree between 77 and 88%, a high crystallinity between 88 and 91%, and a maximum degradation temperature of around 350 °C. Moreover, the solvent system was easily recyclable with consistent performance over 3 cycles of re-use. The extraction method is

well-suited for the extraction of crystalline β-chitin and could be integrated into future works in the production process of chitosan.

Keywords: marine biomass; mollusks; gladius; green solvents; recyclable solvents;

1. INTRODUCTION

Global fishing activities have grown continuously over the past 60 years, producing millions of tons of food but also generating millions of tons of low-value by-products [1]. These by-products, however, are increasingly seen as a very interesting source of high value-added compounds as they mostly contain minerals, proteins, and biopolymers, in particular chitin [2,3]. Industrially, chitin is mainly extracted from crustacean shells and squid pens (also known as gladii), available in abundance and at low cost. In particular, the gladius of the species *Loligo vulgaris*, which can contain up to 30% chitin [4] represents an abundant but underutilized source of chitin. The species is commonly fished off the Basque Coast and much of the Northeast Atlantic from Western Sahara, the Mediterranean east of the Baltic, to the North Sea [5,6]. This natural polymer could be an important resource in the future, given the rapid development of bio-sourced and bio-inspired materials for various applications [3,7–9]. Accordingly, there is also a need to develop sustainable methods for the extraction of such compounds [2,10,11].

Chitin is a linear polysaccharide composed of N-acetyl-2-amido-2-deoxy-D-glucose units linked by β(1→4) glycosidic bonds found abundantly across animal (crustacea, mollusks, insects) and fungal kingdoms [12,13]. Accordingly, depending on the origin, there are different chitin polymorphs which each present a unique molecular conformation: α-chitin, β-chitin, and γ-chitin. α-Chitin presents linear chains arranged in an antiparallel manner with strong intra- and inter-sheets hydrogen bonding and is characteristic of crustaceans [14]. β-chitin presents linear chains arranged in a parallel manner with weak intra-sheets hydrogen bonds and is characteristic of mollusks [15]. γ-chitin is a mixture of antiparallel and parallel chains and is principally found in fungi [16]. Due to its unique chemical and physical structure, chitin presents unique properties, namely biocompatibility, antimicrobial activity, biodegradability, and great amenability to chemical and enzymatic modifications (presence of hydroxyl and

acetyl amide groups, and some residual amine groups) being one of the most actively investigated natural polymers [2,12,13,17,18].

In situ, chitin is associated with proteins and minerals, these fractions varying in importance according to the organism in which it is found [19]. Therefore, the extraction of chitin needs several steps of purification such as deproteination, demineralization, and potentially depigmentation and delipidation. Squid pens are a particularly attractive source of β-chitin, as their processing requires milder conditions than crustacean shells. Indeed, the latter are generally highly mineralized (20-60%) [20], requiring a strong acid demineralization treatment [21], whereas squid gladius contain very low amounts of minerals (around 2% or less) [22] and this step may be skipped [15,23]. Several extraction methods have been studied, chemical, biological, or a combination of these different approaches. The conventional chemical method, which is usually utilized in the industry, uses strong alkali and acid treatments to remove respectively the minerals and proteins, resulting in a negative impact on the environment but also on the chemical and physical properties of the chitin [24]. Alternative methods of chitin extraction, including a variety of green methods [25] seeking to reduce the use of strong alkaline and acid reaction media, simplify the process, or tailor the properties of the extracted polymer, have used microbial [26] (for instance, lactic acid fermentation) or enzymatic methods [27] for α- and β-chitin, or recently renewable solvents such as deep eutectic solvents (DES) for α-chitin [2,28,29]. To the best of our knowledge, DES systems have not yet been used to extract β-chitin from any biomatrix in which it occurs.

DES are precise mixtures of two or more molecular species that result in a system with a melting point considerably lower than each composing species taken individually. This is due to the formation of networks of hydrogen bonds, with one species acting as a hydrogen bond donor (HBD) and the other as a hydrogen bond acceptor (HBA). Ultimately, this results in the formation of viscous liquids at room temperature, due to the low lattice energy of the hydrogen

bond networks. DES are closely related to Ionic liquids (ILs) [30] and share many of the advantages of ILs: negligible vapor pressure, high boiling points, large electrochemical window, and the possibility of recycling [31,32]. Where ILs can be difficult to produce and have mixed environmental credentials, DES are generally cheap and easy to produce, do not react with water, and many are readily biodegradable and use non-toxic metabolites as raw materials [33]. Over the past 10 years, DES have been shown to efficiently extract α-chitin and process crustacean by-products, and chitin is the most commonly targeted marine-origin polymer for extraction by DES [34]. Indeed, DES have been shown to solubilize [35], demineralize and remove proteins from chitin matrices [36–38], and even deacetylate α-chitin [39,40]. The most widely used are highly acidic DES, namely Choline Chloride: Malonic acid, and Choline Chloride: Lactic acid showing the best results in highly mineralized crustacean chitin [36–38].

In the present study, for the first time, DES were tested to extract β-chitin from *Loligo vulgaris* (European squid) pens. As the properties of β-chitin have previously been shown to be dependent on the extraction conditions [41], and previous studies have shown acidic type DES are sufficient for the extraction of α-chitin, given the high protein content and low mineral content of squid pen, herein, we tested the efficacy of 6 acidic, neutral or alkaline DES (Table 1).

2. MATERIALS AND METHODS

2.1. Chemicals and Raw Materials

Choline chloride (2-hydroxyethyl(trimethyl) azanium;chloride - $C_5H_{14}ClNO$, ACROS Organics), betaine (2-(triméthylazaniumyle) acetate - $C_5H_{11}NO_2$, Alfa Aesar), dipotassium carbonate (K_2CO_3, Alfa Aesar) malonic acid (propanediolic acid - $C_3H_4O_4$, Alfa Aesar),

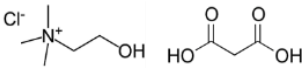
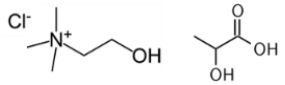
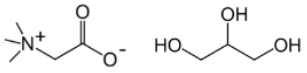
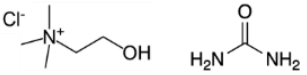
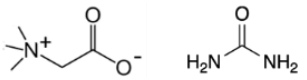
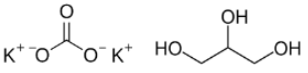
glycerol (propane-1,2,3-triol - $C_3H_8O_3$, Alfa Aesar), urea (CH_4N_2O) and lactic acid (2-hydroxypropanoic acid - $C_3H_6O_3$, 90%) were purchased from Thermo Fisher Scientific (France). All chemicals were reagent grade.

Loligo vulgaris pens (European squid captured in September 2020 in the Atlantic Ocean) were kindly provided by the company Mericq, Saint Jean de Luz, France.

2.2. DES preparation

In this work, the efficacy of 6 DES systems qualified as "acidic", "neutral", or "alkaline" relative to their constituent hydrogen bond acceptors or donors was evaluated (Table 1). The DES were formed by accurately weighing out and combining individual components under magnetic stirring at 80 °C for 2 h. Excess moisture was removed by placing the mixture in a rotary evaporator (BUCHI R-100; Flawil, Switzerland) at 30 mPA and 50 °C until constant weight.

Table 1: DES used in this study: type, system abbreviations, molar ratios, and structure.

pH	DES system	HBA	HBD	Molar Ratio (HBA: HBD)	Structure
Acidic	**CCMA**	Choline Chloride	Malonic Acid	1:2	
	CCLA	Choline Chloride	Lactic Acid	1:2	
Neutral	**BeGLY**	Betaine	Glycerol	1:2	
	CCU	Choline Chloride	Urea	1:1	
Alkaline	**BeU**	Betaine	Urea	1:1	
	KGLY	Potassium carbonate	Glycerol	1:5	

2.3. Chitin extraction

Figure 1 shows the chitin extraction process and DES recycling approach used in this study.

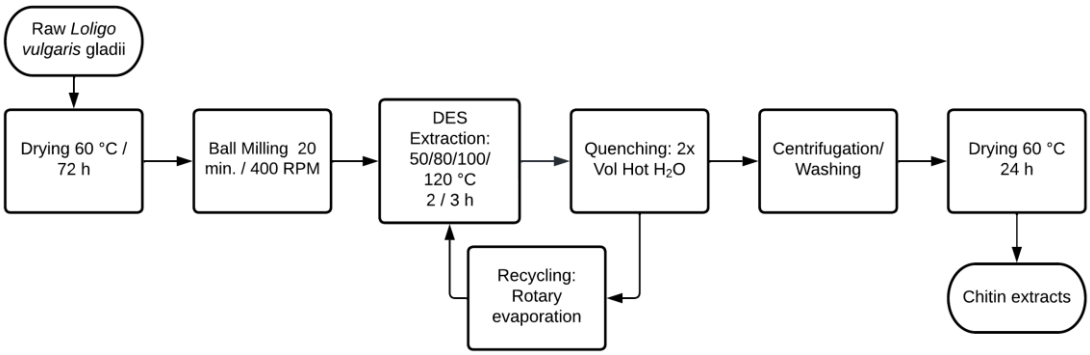


Figure 1: Overview of the chitin extraction process and DES recycling.

The *Loligo vulgaris* pens were washed with warm tap water to remove surface contamination and then dried at 60 °C for 72 h in a circulating air oven. The dried gladii were then powdered to uniform 0.2 mm diameter particles using a RETSCH PM100 ball mill (Haan, Germany) for 20 min at 400 rpm, and conserved at 4 °C until extraction. For the extraction, the powdered squid pens were introduced into 100 mL screw cap vials with the DES in a 1:25 w/w ratio and the mixture was placed in a glycerol bath and heated to 50, 80, 100, or 120 °C for 2 or 3 h under magnetic stirring (400 rpm). The DES BeU, which is solid at 50 °C, was only used at higher temperatures. To quench the reaction, twice the solution volume of hot water was added to the vial, and the mixture was centrifuged at room temperature for 5 min at 4000 rpm to separate the chitin. The extract was then washed abundantly with hot (~ 80 °C) distilled water until pH reached neutrality. The extracts will be referred to according to the DES, the extraction temperature, and time, *i.e.*, the *Loligo vulgaris* extract obtained with KGLY at 100 °C for 2 h will be called KGLY100-2. For comparison, a conventional alkaline extraction (abbreviated here as ALC) was also performed using a method from Lavall [15] with slight modifications. Briefly, squid gladius powder was mixed with 1 M NaOH for 2 h with a 1:30 w/v ratio at 80

°C, washed and centrifuged until the pH of the solution reached neutrality. All extracts were dried overnight at 60 °C before the determination of yield, then conserved in the dark at room temperature before further analysis.

To assess solvent recyclability, for selected conditions, the supernatant from the quenching step (Figure 1) was dried using a rotary evaporator at 50 °C and 30 mPa. Once constant weight of the solution was attained, fresh *Loligo vulgaris* pens powder was introduced at the same ratio as initially (1:25, w/w), and extraction was repeated in the same conditions as described before. After each sequential step, the isolated chitin extracts were separated, dried, and stored as above. Yield determination, CHN elemental analysis, ATR-FTIR, and TGA were used to evaluate the efficacy of recycling.

2.4. Samples characterization

2.4.1. Extraction yields determination

The yields of the ensuing β-chitin samples were calculated according to Equation 1:

$$\text{Yield } (\%) = \frac{\text{Final mass}}{\text{Initial mass}} \times 100 \qquad \text{Eq. 1}$$

2.4.2. CHN Elemental Analysis (EA)

A FLASHEA 1112 Elemental Analyzer (Delft, Netherlands) was used to determine the carbon (C), nitrogen (N), and hydrogen (H) contents of extracts with measurements performed at least in duplicate. The equipment was calibrated using 2,5-bis(5-tert-butyl-benzoxazol-2-yl)-thiopen (BBOT, $C_{26}H_{26}N_2O_2S$, Thermo Fisher, France) and 4-aminobenzenesulfonamide (sulphanilamide, $C_6H_8N_2O_2S$, Thermo Fisher France) standards in linear calibration mode. 1.5 mg samples were mixed with 5 mg vanadium pentoxide and incinerated at 900 °C on a He carrier gas. Eager 300 software was used to obtain CHN chromatograms and relative percentages of each atom.

The degree of acetylation was determined from the carbon/nitrogen ratio (C/N). This ratio varies from 5.145 in completely N-acetylated chitosan to 6.861 in chitin, the fully N-acetylated biopolymer ($C_8H_{13}O_5N$ repeat unit). The degree of acetylation (DA) was calculated according to Equation 2 [42]:

$$DA = \frac{\frac{C}{N} - 5.145}{6.861 - 5.145} \times 100 \quad \text{Eq. 2}$$

2.4.3. Attenuated Total Reflectance- Fourier Transform Infrared (ATR-FTIR) spectrophotometry

ATR-FTIR spectra of the extracts were obtained using a ThermoScientific Smart iX Nicolet iS20 infrared spectrometer (ThermoScientific, Waltham, USA) equipped with a KRS-5 crystal (refractive index 2.4; incidence angle 45°). The spectra were recorded in the transmittance mode between 550 and 4000 cm^{-1}, with a resolution of 4 cm^{-1}, and after 128 scan accumulations.

2.4.4. Scanning Electron Microscopy

Scanning electron microscopy images were taken using a HIROX SH-3000 (France) after metallization with gold under vacuum at 30 mA for 60 seconds using a DESK V (DENTON VACUUM, USA). The images were collected with a voltage of 25 kV.

2.4.5. X-Ray Diffractometry

X-ray powder diffraction patterns were collected by using a Philips X'pert PRO automatic diffractometer (Phillips N.V., Amsterdam, Netherlands) operating at 40 kV and 40 mA, in theta-theta configuration, secondary monochromator with Cu-Kα radiation (λ = 1.5418 Å) and a

PIXcel solid state detector (active length in 2θ 3.347º). Data were collected from 5 to 70° 2θ, step size 0.039º, and time per step of 350 s at room temperature (scan speed 0.029º/s).

Crystallinity Index (C.I) was calculated based on Equation 3 according to the method of Sagheer et al. [43] :

$$C.I.(\%) = \frac{I_{110} - I_{am}}{I_{110}} \times 100 \quad \text{Eq. 3}$$

where:

I_{110} is the maximum intensity (a.u.) of the 110 crystallographic plane; and I_{am} represents the maximum intensity of the amorphous portion diffraction, which usually is found about 2θ = 12.5º-13.5º.

2.4.6. Thermogravimetric Analysis

Thermogravimetric analysis (TGA) was carried out on dried samples using a TA Instruments TGA Q50, Mettler Toledo (USA) at a heating rate of 10 °C min^{-1} to 600 °C in nitrogen atmosphere over a temperature range from 30 to 600 °C. Sample weights were 8 mg, and the flow rate was 20 $mL.min^{-1}$.

3. RESULTS AND DISCUSSION

3.1. Assessment of the best extraction conditions for β-chitin

Herein, the potential of 6 DES systems with acidic, neutral, or alkaline pH was evaluated for their ability to extract β-chitin using different reaction conditions (at 50, 80, 100, or 120 °C for 2 or 3 h). The feasibility of the different DES systems was assessed in a first stage by the determination of the extraction yields, elemental analysis (EA), and ATR-FTIR spectra analysis of the β-chitin-based extracts; and in a second phase by assessing the crystallinity, thermal stability, and morphology of the selected β-chitin samples.

3.1.1. Extraction yields, elemental analysis, and ATR-FTIR spectra analysis of the β-chitin-based extracts

The extraction yields of the different β-chitin extracts obtained with the DES are displayed in Figure 2 and compared with the conventional alkaline treatment (ALC - dotted line).

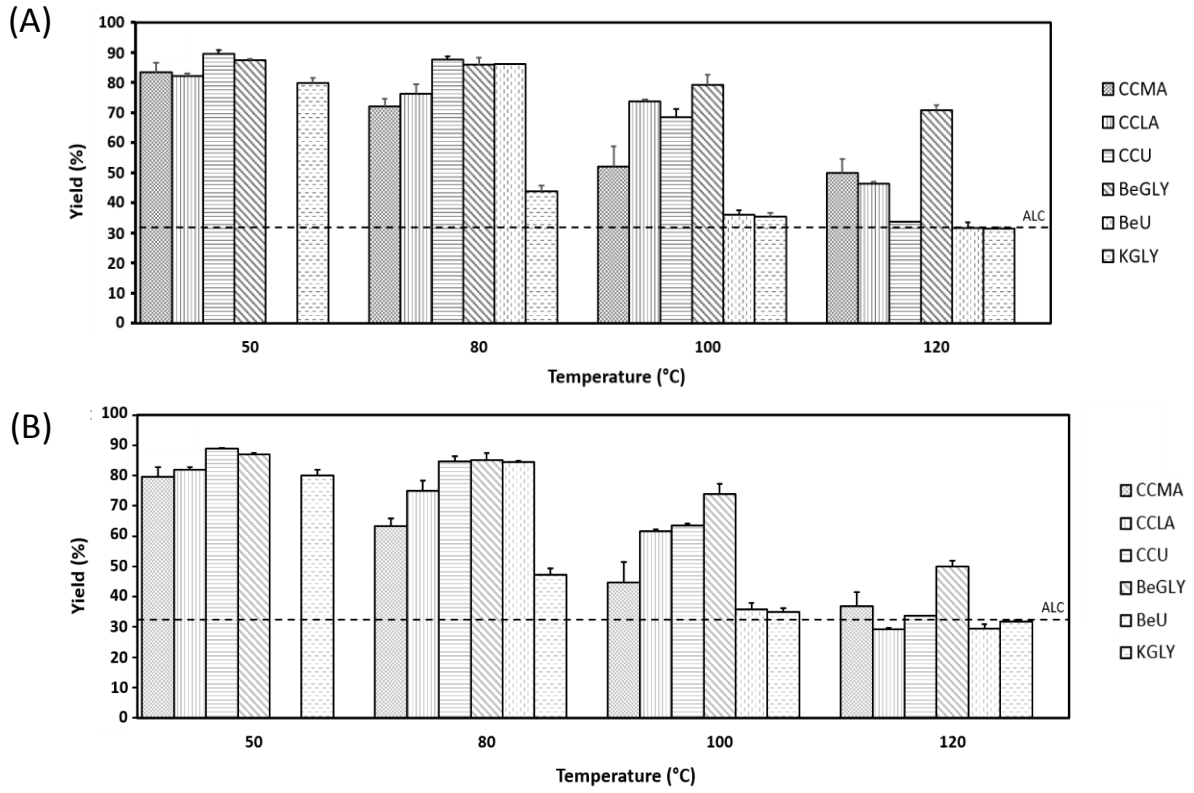


Figure 2: Extraction yields of the obtained β-chitin-based extracts using the different DES systems in function of the temperature for (A) 2h; and (B) 3h. Comparison with the conventional alkaline treatment (ALC - dotted line).

The yield of the β-chitin extracted with the conventional method (ALC) was 32.3 ± 1.2% and was similar to that of Abdelmalek et al. (31.2%) that was obtained via a two-step enzymatic protein removal and demineralization process [4]. In general, for the extraction using acidic and

neutral DES, the results showed extraction yields mostly ranging from 30 to 90% after 2 or 3 h, most of them much higher than the conventional alkaline extraction yield of 32.3%, whatever the temperature tested. The yield values that are closest to the conventional extraction with the acidic or neutral DES were obtained at 120 °C with CCLA after a 3 h extraction (29.2%) and CCU after 2 and 3 h (33.8 and 33.7%, respectively). Nonetheless, the aspect of the extract obtained with CCLA showed a thick hardened crust on top of the chitin powder and a reddish hue. These results indicate that these categories of DES were not able to efficiently purify the chitin and that proteins or residual minerals (this tissue is not mineralized, presenting only 2.8% inorganic material [44]) were still present in the extracts.

On the other hand, the results obtained with the alkaline DES showed that for temperatures lower than 100 °C, the yields of β-chitin extracts were also much higher than that of the conventional alkaline extraction *i.e.*, between 45 and 80%, with similar results for a 2 or 3 h extraction. The results obtained for the extractions at 100 and 120 °C using alkaline DES were, however, more interesting. Indeed, the β-chitin extract yields reached 31.7 and 29.4% with BeU at 120 °C after 2 and 3 h of extraction, respectively, and between 31.5 and 34.9% with KGLY for 2 and 3h extractions at 100 or 120 °C, respectively, which is similar to what was obtained with the conventional alkaline extraction. For all of the DES studied in this work, lower temperatures were insufficient to produce high-purity extracts. This result was different from what was observed in other studies, where 50 °C was enough to obtain α-chitin [38,45,46].

The ten β-chitin-based extracts with the yields closest to that of the conventional alkaline extraction (ALC) were selected for further analysis, namely ATR-FTIR (Figure 3) and CHN elemental analysis (Table 2), in order to assess any structural modification occurring during the extraction or residual impurities remaining in the β-chitin-based extracts.

The ATR-FTIR spectroscopy was carried out to assess the efficacy of the selected DES systems by observing the characteristic bands of β-chitin and detecting potential residual impurities (Figure 3).

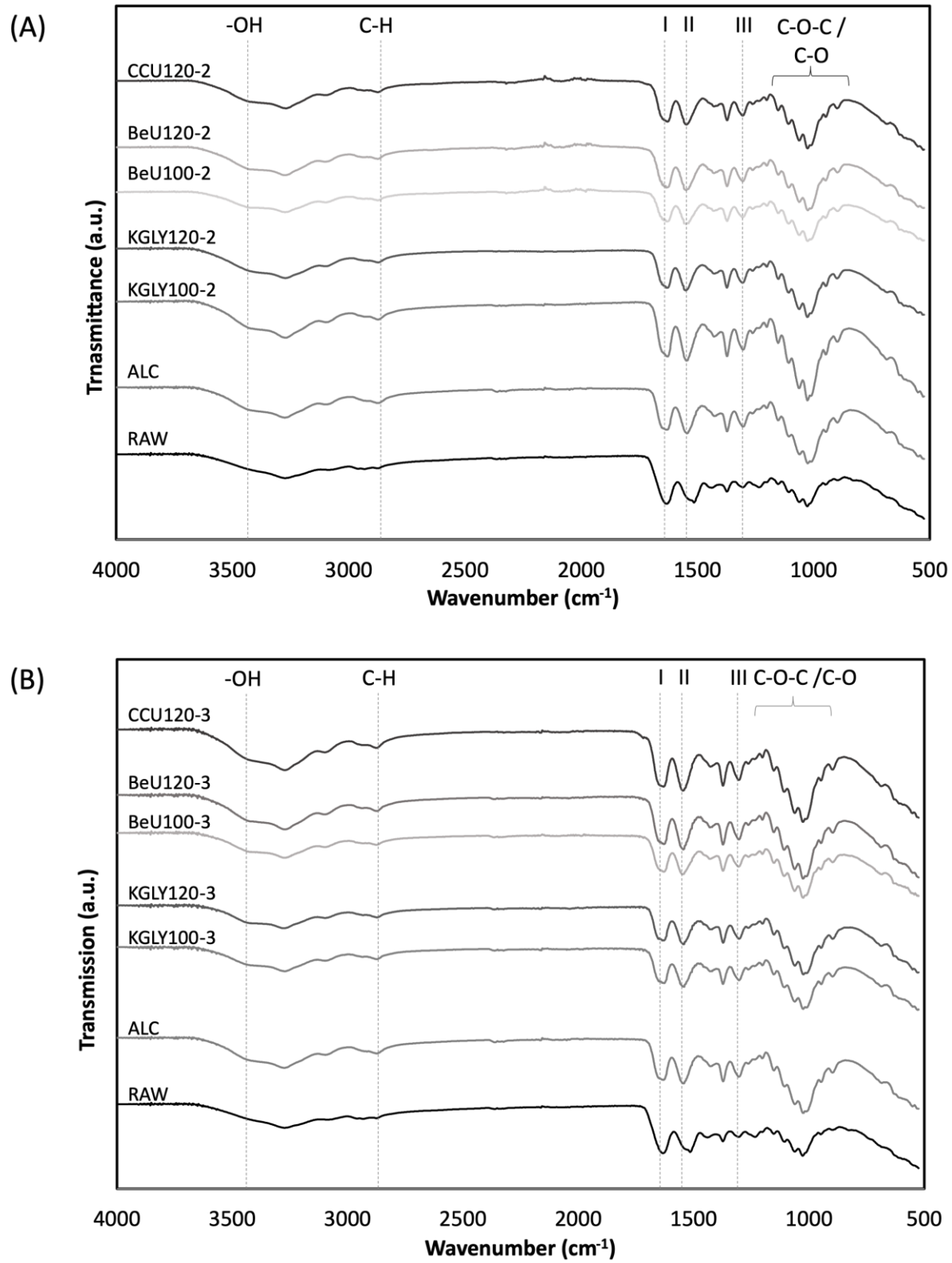


Figure 3: ATR-FTIR spectra of the β-chitin-based extracts obtained with the previously selected DES systems for (A) 2h; and (B) 3h. Note: Roman numerals indicate amide bands (I, II, and III).

As indicated in Figure 3, characteristic bands of β-chitin were found for ALC and all selected β-chitin extracts, *i.e.*, a broad band at 3420 cm^{-1} attributed to the O-H stretching vibrations; a band at 2875 cm^{-1} corresponding to the C-H stretching vibrations; a unique single band at 1630 cm^{-1} corresponding to amide I characteristic of the β-structures; a band at around 1550 cm^{-1} assigned to the amide II; a band at 1420 cm^{-1} corresponding to the CH_2 bending and CH_3 deformation; a band at 1310 cm^{-1} corresponding to the amide III; and multiple peaks between 1150-950 cm^{-1} assigned to the C–O stretching. These bands have all been previously observed in the β-chitin from *Loligo vulgaris* and in other squid genera [4,47,48]. ATR-FTIR spectra also showed key differences in the performance of the DES in extracting β-chitin regarding the presence of other bands that are not characteristics of β-chitin. For instance, it is possible to observe a band at around 1745 cm^{-1} corresponding to the C=O stretching vibrations in the samples extracted with CCU. This demonstrates that probably, this DES formed strong interactions (e.g., H bonds) with target molecules making them difficult to remove, or there is some residual proteins in the extracts [2,34].

After this analysis of the extracts, to better consider the efficacity of the DES, carbon/nitrogen ratios (C/N) were obtained by elemental analysis (CHN) and are shown in Table 2. C/N ratio varies from 5.14 in chitosan to 6.86 in chitin, the fully N-acetylated biopolymer. Among the 10 selected β-chitin-based extracts, KGLY100-3, KGLY120-2, and KGLY120-3 showed C/N ratios of 6.60, 6.48 and 6.66 and %N of 6.48, 6.64, and 6.46%, respectively, which was very close to that of the theoretical pure chitin with a C/N ratio of 6.86 and a %N of 6.89% [42]. These results confirmed the effectiveness of these specific alkaline DES to extract purified β-

chitin. The other β-chitin-based extracts exhibited C/N ratios and %N very far from the theoretical values (Table 2). The high %N values demonstrated the presence of residual proteins in the samples. Concerning the %C, in general, it was observed that the values were slightly lower than theoretical values, although this is commonly observed in CHN analysis of chitin samples of different origins [49–51]. Conventional alkaline treatment ALC also resulted in high purity β-chitin, confirming the findings of previous studies [15,23,47].

Table 2. CHN elemental composition of the selected β-chitin-based extracts, and ALC. Data are the mean ± SD.

Sample Identification	**C/N**	**N** (%)	**C** (%)	**H** (%)
CCU120-2	5.69 ± 0.02	7.59 ± 0.14	43.20 ± 0.65	6.35 ± 0.14
CCU120-3	5.90 ± 0.03	7.24 ± 0.12	42.74 ± 0.46	6.50 ± 0.30
BeU100-2	5.28 ± 0.03	8.36 ± 0.01	44.16 ± 0.22	6.54 ± 0.09
BeU100-3	5.42 ± 0.03	8.12 ± 0.08	44.09 ± 0.19	6.46 ± 0.26
BeU120-2	5.56 ± 0.09	7.76 ± 0.15	43.15 ± 0.13	6.57 ± 0.08
BeU120-3	6.23 ± 0.07	6.72 ± 0.03	41.90 ± 0.41	6.35 ± 0.21
KGLY100-2	6.18 ± 0.08	7.06 ± 0.09	43.61 ± 0.00	6.92 ± 0.04
KGLY100-3	6.60 ± 0.12	6.48 ± 0.17	42.99 ± 0.51	6.50 ± 0.17
KGLY120-2	6.48 ± 0.08	6.64 ± 0.12	43.03 ± 0.21	6.58 ± 0.39
KGLY120-3	6.66 ± 0.11	6.46 ± 0.18	43.05 ± 0.73	6.76 ± 0.20
ALC	6.84 ± 0.02	6.25 ± 0.05	42.58 ± 0.18	6.68 ± 0.09
CHITIN*	6.86	6.89	47.29	6.45

**Chitin, theoretical values from 100% acetylated chitin from Kasaai et al.* [41]

Based on the yields, elemental analysis, and ATR-FTIR spectra, KGLY100-3, KGLY120-2, and KGLY-120-3 showed the closest properties to those of β-chitin, and were selected for

further characterization, namely the determination of the degree of acetylation (DA), morphology, crystallinity and thermal properties, and recyclability tests.

3.1.2. Characterization of the β-chitin samples by SEM, X-Ray Diffraction, and TGA

The degree of acetylation was determined by EA using Eq. 2 and the values are listed in Table 3, which summarizes the properties of the conventional and DES extracted β-chitin samples. The low DA values of the β-chitin extracted with the DES systems (between 77.6 and 88.6%) compared to the ALC one (98.6%) could be justified by some deacetylation of the acetyl groups in C2 of the chitin backbone occurring during the extraction process under the action of the alkaline DES and the high reaction temperatures used [52].

Table 3. Summary of the general properties of the β-chitin samples obtained with the conventional alkaline process and the DES systems.

Sample	**Yield** %	**C/N**	**DA** %	**2Θ** ° (010)	° (1-10)	**CI** %	**Td_{max}** °C
ALC	32.3 ± 1.2	6.8	97.5 ± 1.7	8.3	19.7	84.3	349.5
KGLY100-3	34.9 ±1.5	6.6	85.0 ± 7.0	8.2	19.9	86.7	349.3
KGLY120-2	31.5 ± 0.9	6.5	77.6 ± 4.7	8.3	19.6	91.2	345.6
KGLY120-3	31.8 ± 1.0	6.7	88.6 ± 6.5	8.3	19.7	88.3	347.4

SEM was used to assess the morphology of the ensuing β-chitin powder samples, and no major morphological differences were observed as displayed in Figure 4. The samples all presented a

lamellar structure similar to that of unprocessed *Loligo vulgaris* gladius powder (A). This observation is in line with previous works [53].

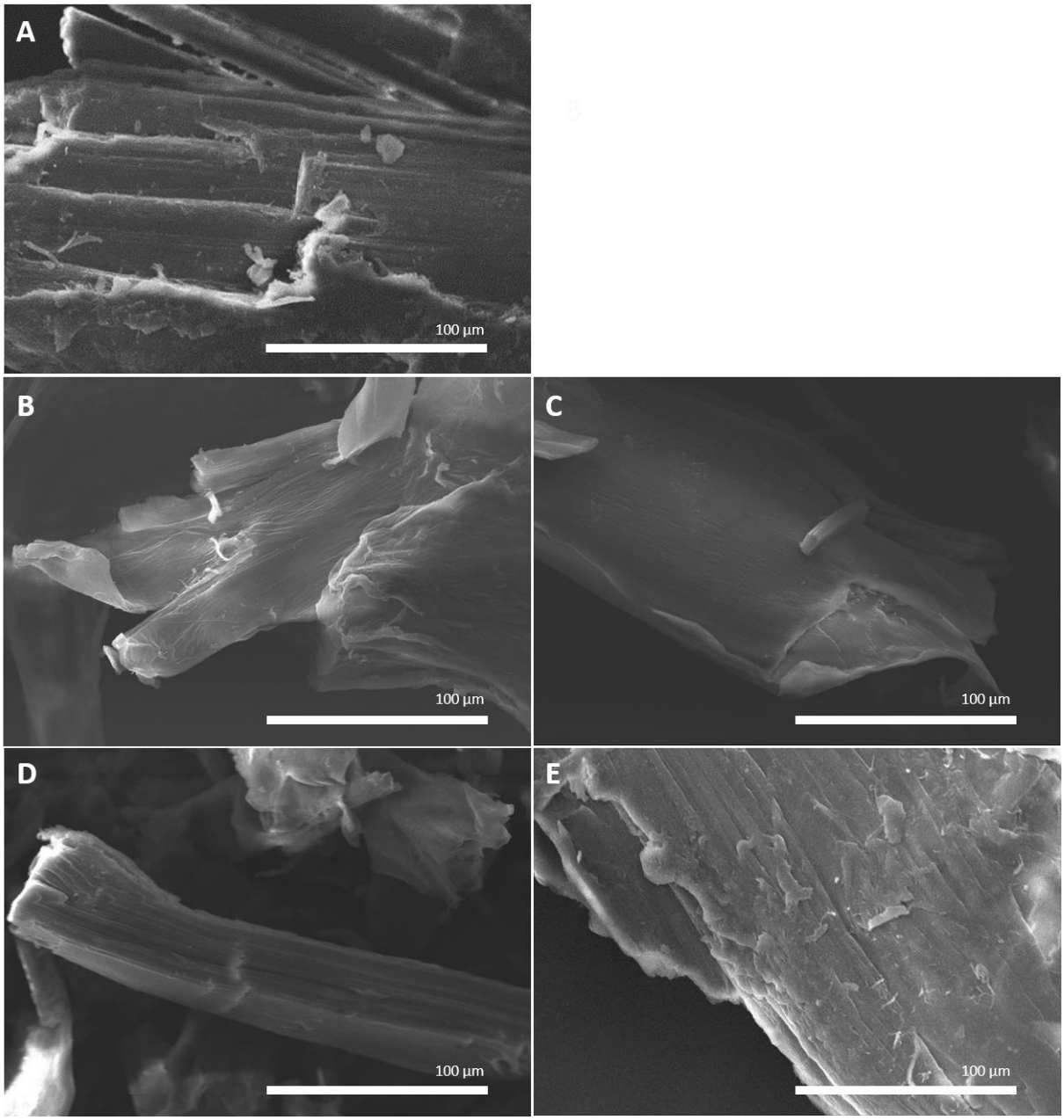


Figure 4: SEM pictures (x 500) of: (A) *Loligo vulgaris* (raw gladius powder) (B) ALC; (C) KGLY100-3; (D) KGLY120-2; and (E) KGLY120-3 powders.

The crystalline structure of the β-chitin, extracted with the conventional alkaline method or with the alkaline DES, was analyzed by X-ray diffraction. The diffractograms are shown in Figure

5. They exhibit the typical diffraction pattern of β-chitin in which 2 important crystalline reflections were observed between the ranges 5-80 in 2θ, which is in line with the characteristic Miller indices of the diffraction peaks of chitin [54].

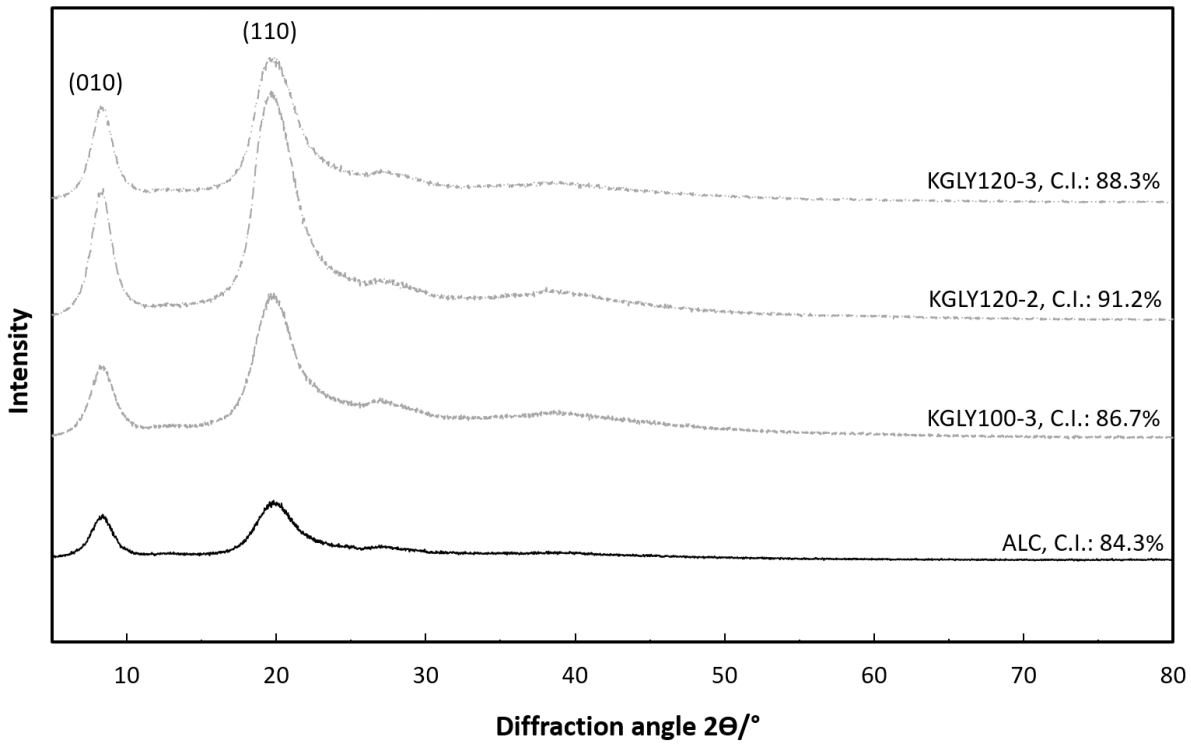


Figure 5: X-ray diffraction (XRD) patterns and crystallinity index (C.I. %) of β-chitin samples extracted with the alkaline DES and the traditional alkaline method (ALC).

Indeed, the diffractograms of the β-chitin samples by the alkaline DES displayed a peak around 8.4° and another around 19.8°, corresponding to the crystal plane (010) and (110) planes [55,56], respectively. These results are similar to what was obtained for the β-chitin extracted with the ALC method (Figure 5 and Table 3). These patterns were also observed by Kaya et al. [16] regarding *L. vulgaris* chitin, and Hajjis et al. [57] with β-chitin from *Sepia officinalis.* Interestingly, the crystallinity index (C.I.) of DES-extracted chitins was higher than that of the alkaline treatment, with C.I. of 88.3-91.2% against 84.3% for the traditional method. The C.I. obtained in the study was very close to that of squid pen β-chitin Férnández-Martin et al. [10]

(around 85%) and *Illex argentinus* squid pen by Wu et al. [58] (88.9%). The high crystallinity of the β-chitin extracted with DES compared to the conventional alkaline one may be due to the removal of amorphous regions under the action of the bulkier DES and the high reaction temperatures used. High crystallinity being desirable for the synthesis of chitin biomaterials [59], this result could be promising for further material development.

Figure 6 shows the thermogravimetric (TGA, A) and derivative (dTGA, B) thermogram profiles of the raw material (squid pen) and obtained β-chitin over the temperature range of 30 to 600 °C under a nitrogen atmosphere.

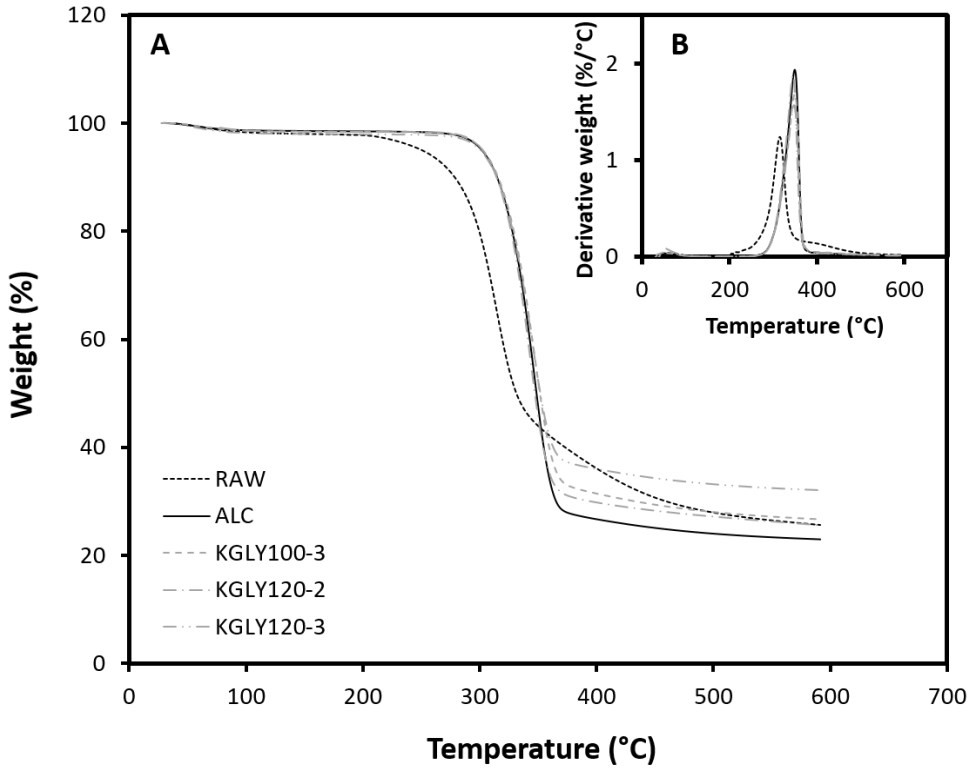


Figure 6: TGA (A) and dTGA (B) thermograms of raw material (squid pen) β-chitin extracted with alkaline DES and traditional alkaline extraction.

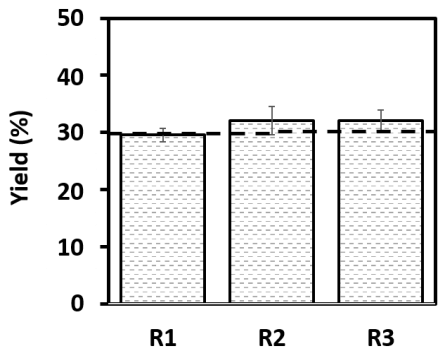
The first mass loss observed in all samples at around 100 °C (Figure 6) corresponds to the evaporation of absorbed and bound moisture [60]. The second mass loss, and the most

important, was observed at around 305 and 350 °C for the raw material (RAW) and the β-chitin extracted samples, respectively. In the case of the extracted β-chitin samples, this second mass loss was assigned to the maximum degradation temperature (Td_{max}) of the chitin macromolecule caused by the dehydration of the saccharide structure followed by the decomposition of the acetylated and deacetylated units [61]. The lower Td_{max} of the RAW was attributed to the presence of proteins that present lower temperature of degradation compared with chitin. The Td_{max} of the different samples are listed in Table 3 and are in concordance with those of previous studies [10,16,62]. The RAW and all chitin samples presented a remaining mass of around 20-35%, corresponding to residual inorganic matter and carbonized chitin [63]. Under the conditions used, the samples extracted with KGLY120-3 showed the highest residual percentage.

3.2. Recyclability of the alkaline DES

In order to develop a more environmental and economical approach, the recyclability of the DES system KGLY at 120 °C for 2 h of extraction was examined, as these conditions provided rapidly high-purity chitin. Using these conditions, it was possible to recycle this particular DES up to 3 times without any significant variations of the yield (around 30%, Figure 7 A) and C/N ratio (6.45-6.52, Figure 7 B) when compared to the first extraction. Also, no important changes were observed after each recycling cycle in terms of β-chitin properties, *i.e.*, DA (76.1%-78.8%, Figure 7 B), chemical structure (ATR-FTIR spectra, Figure 7 C), and thermal stability (Td_{max} around 350 °C, Figure 7 D).

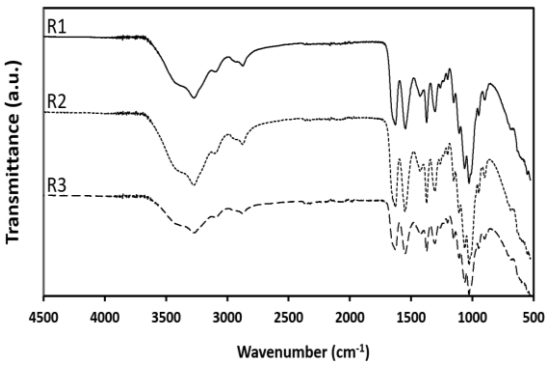
(A)



(B)

Sample	R1	R2	R3
C/N	6.45 ± 0.02	6.52 ± 0.03	6.47 ± 0.15
N	6.64 ± 0.03	6.58 ± 0.02	6.87 ± 0.08
C	42.85 ± 0.07	42.87 ± 0.01	44.47 ± 1.54
H	6.59 ± 0.22	6.78 ± 0.11	6.76 ± 0.01
DA (%)	76.1 ± 1.3	77.1 ± 1.6	78.8 ± 8.7

(C)



(D)

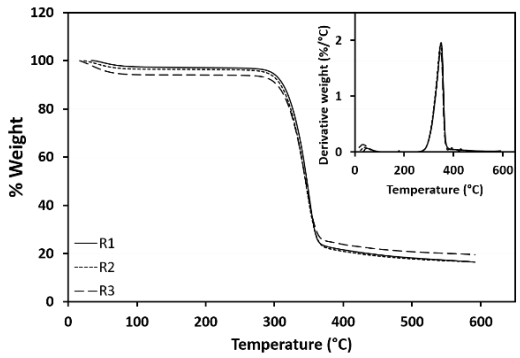


Figure 7: Recycling viability: (A) % yields (comparison with the first extraction (dotted line)); (B) Elemental analysis and DA, data are mean ± SD; (C) ATR-FTIR spectra; and (D) TGA and dTGA thermograms of the β-chitin extracted with three successive cycles using KGLY at 120 °C for 2h. R1: First recycling, R2: Second recycling, R3: Third recycling.

Recycling was discontinued after the third round, as the solvent was excessively viscous and its recuperation became very difficult.

Moreover, the simplicity of the procedure, requiring no additional alkaline or precipitation agent is worthwhile to consider.

4. CONCLUSION

In the present work, β-chitin from *Loligo vulgaris* pens was extracted for the first time with a simple DES solvent system, resulting in high purity and crystalline chitin with a yield of around

30%. Six acidic, neutral, or alkaline DES systems were tested, from 50 to 120 °C, with extraction times of 2 and 3 h, and compared with a conventional alkaline extraction. In the first part of the study, the yield, elemental analysis, and ATR-FTIR spectra showed that, among the DES that were tested, the alkaline potassium carbonate: glycerol (KGLY) was the most efficient one compared to the acidic and neutral DES systems and that the highest temperatures of 100 and 120 °C for 2 or 3 h were necessary to obtain purified β-chitin. The selected β-chitin samples were further characterized in terms of morphology, crystallinity, and thermal properties. The results showed that KGLY led to high quality β-chitin, similar to that obtained with the conventional alkaline extraction. Additionally, KGLY could be re-used three times without affecting the quality of the β-chitin. Because of the high purity and crystallinity of the obtained β-chitin, as well as the possibility to be recycled, the use of the alkaline KGLY DES was shown to be a particularly suitable method to isolate β-chitin and, potentially, for chitosan production.

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Author Contribution

Conceptualization: Colin McReynolds, Amandine Adrien, Susana C.M. Fernandes; Methodology: Colin McReynolds, Amandine Adrien, Susana C.M. Fernandes; Formal analysis and investigation: Colin McReynolds, Nelly Brugerolle de Fraissinette, Sheila Olza; Writing - original draft preparation: Colin McReynolds, Amandine Adrien; Writing - review and editing: Colin McReynolds, Nelly Brugerolle de Fraissinette, Sheila Olza, Amandine Adrien, Susana C.M. Fernandes; Funding acquisition: Susana C.M. Fernandes; Supervision: Susana C.M. Fernandes.

Competing Interests Statement

Not applicable.

Data Availability Statement

Not applicable

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