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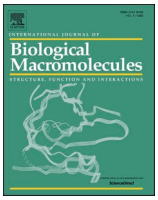

Isolation and quantification of alginate in choline chloride-based deep eutectic solvents

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ABSTRACT

Extraction of seaweed compounds using Deep Eutectic Solvents (DES) has shown high interest. Quantification, however, is challenging due to interactions with DES components. In this research work, three chemical separation techniques were investigated to isolate and quantify alginate from a set of choline chloride-based DES. While choline chloride served as the hydrogen bond acceptor (HBA); Urea, Ethylene Glycol, Propylene Glycol, Glycerol, Sorbitol, Xylitol and Glucose were used as hydrogen bond donors (HBD). DES containing sodium alginate were subjected to precipitation with sulfuric acid 0.2 M (pH 1.6), ethanol-water mixture (80 % *v*/v) and calcium chloride (1 % *w*/*v* $CaCl_2{\cdot}2H_2O$). Alginate in precipitates was quantified and used to evaluate the performance of each separation technique. The highest recovery yields (51.2 ± 1.3 %) were obtained using the ethanol-water mixture followed by calcium chloride (45.7 ± 1.2 %), except for polyols (e.g. sorbitol). The lowest recovery yields were obtained with acid, with a particularly low recovery yield when urea was used as HBD (9.6 ± 1.3 %). Estimations of ManA/GulA ratios showed lower values for precipitates from DES compared to the ones obtained from water. This research shows ethanolic precipitation as a suitable method for alginate separation from the studied set of choline chloride-based DES.

1. Introduction

Alginate, a phycocolloid, represents a relevant biomaterial with a broad set of current and potential applications. It is used as a thickener and as a gelling agent in the food industry; or within the medical sector, as a coating material for improving drug delivery and for the production of dental imprints [1–5]. This phycocolloid is obtained from brown seaweed (*Phaeophyceae*), a group of macroalgae that differentiates for its colour due to the presence of fucoxanthin, a high-valued xanthophyll. Unlike plants, brown algae accumulate energy by synthesizing laminarin (a glucan) and mannitol, relevant carbohydrates with potential industrial value [6–8]. Together with a sulphated fucose-based polymer called fucoidan, alginate could be found attached to the cell wall forming salts with sodium calcium and magnesium, among other divalent cations. It provides the algae with the mechanical strength and flexibility needed for the harsh environment that the organism is exposed to. It could represent up to 40 % of the dry weight of the seaweed although this value varies dramatically among species and seasons of collection [6,9].

Alginate is an anionic linear polysaccharide consisting of randomly distributed blocks of (1–4)-linked *β*-D-mannuronic acid (M) and α-L-guluronic acid (G), both as pyranoses [10,11] (Fig. 1). The distribution and proportion of these two monomers is variable and depends on the species, part of the seaweed and even the season when it is collected [12]. These proportions determine the techno-functionalities of the alginate and its potential applications. While the length of the polymer normally determines the viscosity of alginate solutions. D-mannuronic and L-guluronic acid ratios (M/G) and the distribution of M and G units influence the strength of gels formed under the presence of multivalent ions such as Ba^{2+}, Sr^{2+}, Ca^{2+} and Co^{2+} [10,13]. The higher the proportion of L-guluronic acid and the longer the G blocks, the stronger the formed gel. These characteristics are highly valuable in the industry and determine the quality of the alginate.

The alginate extraction process comprises several steps with different basic or acid chemical additions. Additionally, other chemicals such as paraformaldehyde could be added to remove pigments and for seaweed preservation to avoid the costly drying process [6,14,15]. One of the common processes comprises alkali precipitation using sodium carbonate. Seaweed is acidified to remove divalent cations such as Ca^{+2} and Mg^{+2} which results in seaweed containing alginic acid. Sodium

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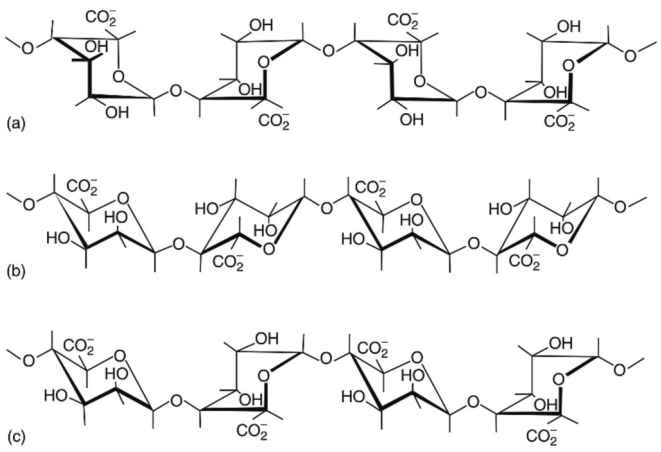

Fig. 1. Structure of alginate: a) chain of L-guluronic acid (G); b) chain of D-mannuronic acid (M); c) alternated L-guluronic and D-mannuronic acids. Reproduced from Rinaudo [10].

carbonate is added producing a viscous seaweed solution which is diluted with large amounts of water and physically separated from the seaweed debris. Alginate is then recovered in the form of fibres by reducing the pH of the solution mixed with sodium carbonate and converted again into sodium alginate, which is finally dried and milled. Other routes like precipitation with ethanol or calcium chloride are applied as well [6,15,16].

Large amounts of water are used for better handling the viscous alginate solution that is formed during the extraction process. It has been reported that the water used to produce one ton of sodium alginate ranges from 1000 to 1500 m^3 [17]. Considering that worldwide the production of commercial alginate is estimated at 30,000 metric tons per year. The amounts of water, the harsh extraction conditions and the presence of harmful chemicals such as paraformaldehyde, make the process unsustainable [15].

One important green technology recently explored for the valorisation of bio-compounds more sustainably, is the utilization of Deep Eutectic Solvents (DES). These types of solvents are defined as a mixture of two or more components: Brønsted-Lewis bases and acids. They possess high melting points individually but when mixed the melting point of the mixture decreases dramatically. Normally composed of a Hydrogen-bond acceptor (HBA) and a Hydrogen-bond donor (HBD). Hydrogen bonds between the compounds determine their good solvent properties [18,19]. Deep eutectic solvents have drawn attention since they can be synthesized from natural and biodegradable compounds, the so-called Natural Deep Eutectic Solvents (NaDES). Eutectic mixtures could be obtained from mixing ammonium quaternary salts such as choline chloride with amides, polyols, sugars, organic acids, amino acids, etc. This makes their manufacture potentially more sustainable and inexpensive. Additionally, as many combinations could be achieved and with different proportions of each compound, a DES can be tuned to provide the optimal conditions for the target (s) compound extraction [20].

Several investigations have been conducted to extract compounds such as lipids, pigments, and polyphenolic compounds with potential bioactive applications with relative success [20,21]. However, the extraction of macromolecules represents a bigger challenge due to the complex interactions that could come up with the size and the chemical nature of the molecule. Das et al. [22] evaluated the extraction κ-carrageenan from *Kappaphycus alvarezii* using a set of choline chloride-based DES. Extracts were washed with isopropanol and dried for analysis. Saravana et al. [23] investigated the extraction process of alginate with deep eutectic solvents in combination with subcritical water extraction and used calcium chloride for recovering the alginate. It was found, among others, that 70 % of water was needed to obtain the highest yield (28 % dry basis). Water content was tested in the range of 50 and 70 %. In this range of water additions, the DES structure is likely to be already broken as the hydrogen bonds that exist between the DES components are disrupted by water addition at that level, and free constitutes formed in solution [24]. Thus, to the best of found knowledge, there is no publication related to the extraction of alginate using only pure or low-hydrated (<50 % of water) DES.

To assess and compare extraction processes with different DES, a reliable method for quantification and characterization of the alginate needs to be developed. Colorimetric methods for alginate quantification have been developed [25], which can give relatively good results in water solutions. However, reagents from the method react unexpectedly with some DES compounds, creating non-reliable standard curves nor results (non-shown results). Additionally, this method which relies on carbazole reaction, is susceptible to interference with a broad set of compounds, reducing its applicability [26,27]. Then, the alginate quantification is limited to more accurate techniques such as chromatography. The polymer content is normally determined, based on the analysis of its uronic acids which are released after an acid hydrolysis process [28].

Attempts of direct hydrolysis of the extraction samples could be performed, however, undesired reactions among the DES compounds and the acid used could occur as in the colorimetric method. It is worth mentioning that some choline chloride-based DES have been used as catalysts for degradation and oxidation reactions [29–31]. Besides, care must be taken to maintain the structure integrity of the target molecule for further applications and the analytical procedure should reflect this. Additionally, it has to be considered that the lone presence of a solvent could be relevant for certain reactions [32]. Therefore, the separation of alginate from DES for analytical purposes should be accomplished. Alginate in water solutions could be chemically separated via precipitation with acid, ethanol-water mixture or calcium-rich solutions and the pellet dried for further hydrolysis and analysis [15]. The solution could also be concentrated and freeze-dried, before the analysis. However, DES presents certain challenges for the analytical techniques. Due to their low volatility, they cannot be evaporated and separation via the previous chemical methods has not yet been evaluated [19].

This research work is an effort to evaluate different chemical methods to separate the alginate from DES solutions for quantification purposes and identify potential interactions between the solvent and the biopolymer.

2. Materials and methods

2.1. Experimental design

Chemical separation techniques such as precipitation with acid solution, alcohol-water mixture and calcium chloride were tested. Seven different choline chloride-based DES were prepared and mixed with a volume of water solution of a known alginate concentration (Table 1). All these DES-Alginate solutions were then subjected to the aforementioned alginate precipitation techniques and resulting precipitates were hydrolysed and quantified via chromatography. Alginate in water solution was prepared as a control and subjected to the same precipitation treatments.

Table 1
Choline chloride-based deep eutectic solvents.

Abbreviation	HBD	Molar ratio	Water content
U	Urea	1:2	0
EG	Ethylene glycol	1:2	0
PG	Propylene glycol	1:2	0
Gly	Glycerol	1:2	0
Sor	Sorbitol	1:1	10 %
Xyl	Xylitol	1:1	10 %
Glu	D-Glucose monohydrate	2:1	10 %

2.2. Alginate and DES compounds

Alginic acid sodium salt from brown seaweed (medium viscosity) was acquired from Sigma-Aldrich. A solution of 1 %*w/v* in Milli-Q water was prepared and stored at refrigerated conditions for further preparations. Choline chloride, urea, ethylene glycol, propylene glycol, glycerol, sorbitol, xylitol and D-glucose monohydrate were acquired from Sigma-Aldrich.

2.3. DES preparations

Table 1 displays the set of choline chloride-based deep eutectic solvents prepared for the experiments. This set of DES was prepared by mixing the compounds at their corresponding molar rate. The mixtures were placed in a water bath at 80 °C long enough until the components were visually mixed and no crystal or solid was present. Mixtures were stored at room temperature in airtight conditions until use.

2.4. DES-Alginate solutions

DES containing alginate solutions were prepared. A volume of 8 mL of each DES preparation was mixed with 2 mL of the 1 % w/v sodium alginate water solution and gently agitated in a rotating mixer for 30 min at room temperature until the mixture was completely homogeneous. With these proportions, each DES-Alginate solution contained 2 mg mL^{-1} of sodium alginate. Each DES-Alginate solution was prepared three times and the treatments and quantification were performed for each repetition.

2.5. Alginate separation

To proceed further with the polymer quantification, different separation techniques were tested to recover the alginate from the DES solutions. For the acid, ethanolic and calcium precipitation, 1 mL of DES-alginate solution was placed in a 10 mL glass tube and mixed with 4 mL H_2SO_4 0.2 M (pH 1.6); 4 mL of 80 % *v*/v ethanol-water mixture; and 2 mL of 1 % *w/v* $CaCl_2{\cdot}2H_2O$, respectively. All mixtures were stirred vigorously and centrifuged at 4255*g* for 10 min in a Beckman Allegra-X-30R centrifuge. For each sample, the supernatant was carefully removed and the pellet was washed with each corresponding precipitation solution, either sulfuric acid at pH 1.6, ethanol-water mixture at 80 % *v*/v, or calcium chloride at 1 %. Samples were then stirred and centrifugated once more at the previously mentioned conditions. The new supernatant was then removed and the pellet was dried with nitrogen gas overnight and stored refrigerated for further analysis.

2.6. Alginate hydrolysis and uronic acids quantification

Alginate quantification was performed via the content analysis of D-mannuronic and L-guluronic acids. A volume of 1 mL of 11 M H_2SO_4 was added to each glass tube containing the dried pellets. Tubes were placed in a heating block at 37 °C for 1 h. Afterwards, 5 mL of milli-Q water was added to each tube to reach 1.8 M sulfuric acid. Tubes were placed then again in the heating block at 100 °C for 2 h. Samples were then cooled down to room temperature. An aliquot of 1 mL was taken from each tube, filtered with a 0.22 μm pore size filter and placed in HPLC vials. 10 μL of each sample was injected into an HPLC at 60 °C and in a flow rate of 0.8 mL min^{-1} through a Rezex™ ROA-Organic Acid H+ (8 %) LC column (300 × 7.8 mm) from Phenomenex™ with sulfuric acid 0.008 M as mobile phase. Samples were checked to be at a pH lower than 3.0. A refractive index detector (RID) was used. Results were compared with standard solutions prepared with D-mannuronic acid sodium salt (Sigma-Aldrich™) and L-guluronic acid sodium salt (Biosynth™).

2.7. Calculations

2.7.1. Alginate recovery yield

The alginate recovery yield was calculated based on the sum of D-mannuronic acid and L-guluronic acid found in each sample using Eq. (1).

$$\%Recovery\ Yield = \frac{W_{UA} * F_{pol} * F_{Na}}{W_{NaAlg}} * 100 \quad (1)$$

where W_{UA} is the amount of uronic acids detected in the sample [mg]; F_{pol} is the correction factor due to the addition of a water molecule to the monomer during hydrolysis which is defined as $\frac{MW_{mono}}{MW_{UA}}$, where MW_{mono} is the molecular weight [g mol^{-1}] of a monomer unit attached to the polymer and MW_{UA} is the molecular weight [g mol^{-1}] of a uronic acid monomer isolated from the polymer. F_{Na} is the correction factor due to the presence of a sodium atom attached to each monomer in the original sodium alginate sample which is defined as $\frac{MW_{mono+Na}}{MW_{mono}}$, where $MW_{mono+Na}$ is the molecular weight [g mol^{-1}] of a monomer within the polymer in its sodium salt form. Finally, W_{NaAlg} is the amount of sodium alginate in the original sample [mg].

2.7.2. M/G ratio

M/G ratios were estimated by dividing the amounts of D-mannuronic and L-guluronic acids found in each precipitate after the hydrolysis process.

2.8. Statistical analysis

Data analysis and comparison were performed using ANOVA single-factor test ($\alpha = 0.05$) between the groups corresponding to each precipitation treatment. When statistical significance of difference was found among the treatments, a *t*-test (with a Bonferroni-corrected α) was performed in pairs to determine pair-wise differences.

3. Results

3.1. Alginate recovery yield

An overview of the alginate recovery yield (%) after the application of each precipitation method for each DES solution is shown in Fig. 2. Except for the cases in which sorbitol and xylitol were used, the lowest alginate recovery yields were obtained when the acid solution was used. The lowest recovery yields among these acid treatments were obtained from the solutions prepared with urea (9.6 ± 1.3 %) and ethylene glycol (21.8 ± 0.6 %) while it was slightly higher when propylene glycol was used (33.5 ± 1.2 %). There was no significant difference in the recovery yield when glycerol, sorbitol, xylitol and glucose were used, with values ranging from 24.9 ± 2.2 % to 28.8 ± 3.6 %. The recovery yield obtained using ethanolic precipitation was not significantly different among all DES solutions with a recovery yield ranging from 44.0 ± 3.3 % and 51.2 ± 1.3 %. When calcium chloride was used, only in the cases where sorbitol and xylitol were used the alginate recovery yield was significantly low with values of 3.6 ± 0.7 % and 12.9 ± 1.6 % respectively. The recovery yield was not significantly different among the rest of the DES with values ranging from 40.6 ± 0.7 % and 45.7 ± 1.2 %.

Water was used as a control and recovery yields were compared with the values obtained when DES were used for each precipitation treatment (Fig. 3). When acid precipitation was used the recovery yield from water was significantly higher (57.6 ± 4.7 %). A similar behaviour was observed when calcium chloride was applied as a precipitation method with a recovery yield of 56.4 ± 2.4 %. On the contrary, the alginate recovery yield was lower when the ethanolic precipitation was applied in the water solution, with a value of 21.3 ± 3.4 %.

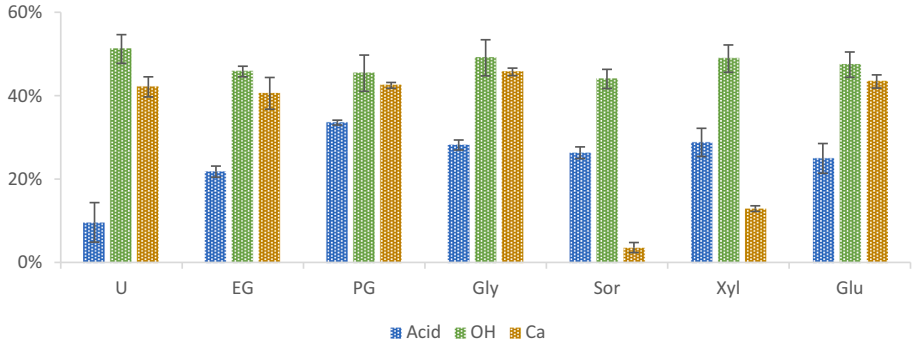

Fig. 2. Obtained alginate recovery yields from the precipitation methods applied to each DES solution.

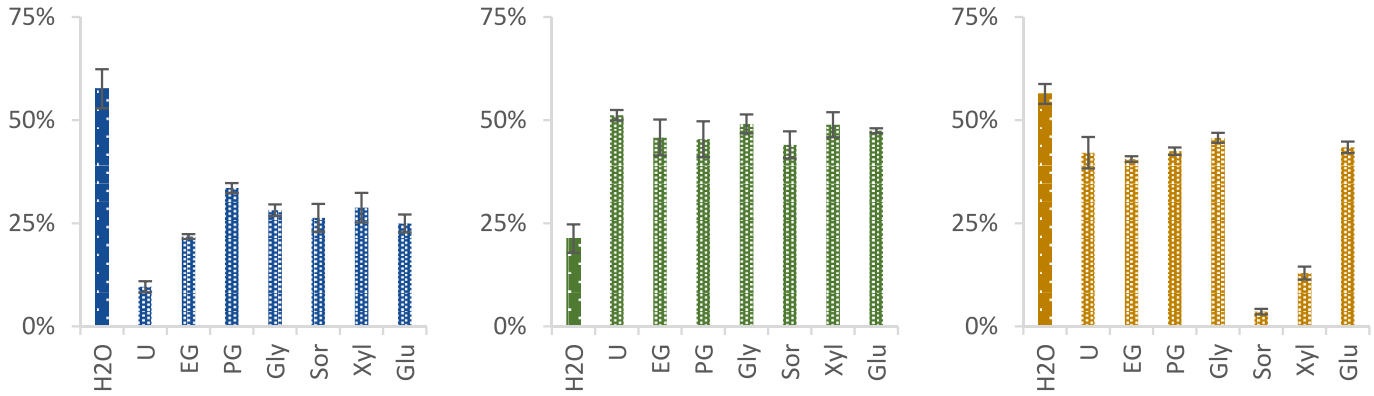

Fig. 3. Alginate recovery yield from each DES solution subjected to acid (left), ethanolic (middle) and calcium (right) precipitation.

3.2. *Alginate precipitates*

After the addition of each precipitation solution, it was observed that the appearance of the new mixtures was changed (Fig. 4). In the control solutions prepared with water, when acid water was added fibres were formed, while a gel-like material was formed when ethanol-water

Fig. 4. Solutions after the addition of acid water, ethanol-water mixture and calcium chloride. From left-top to right-bottom; water, choline chloride-urea, -ethylene glycol, -propylene glycol, -glycerol, -sorbitol, -xylitol and -glucose monohydrate.

mixture and calcium chloride were added. This behaviour was different when the precipitation techniques were applied to the DES solutions. When acid was applied to DES solutions no visible fibres were detected in all the cases, however, a gel-like material was detected after the centrifugation process (Figs. 4 and 5). On the other hand, when the ethanol-water mixture was applied it resulted in a hazy solution and contained observable naked-eye fibres in all DES cases. This was translated into a dust-like material after the centrifugation. When calcium chloride was applied a gel-like material was visible in all the cases, which was also observed after the centrifugation step.

3.3. M/G ratios

M/G ratios were calculated for the alginate precipitates obtained from each DES solution at different precipitation methods (Fig. 6). The M/G ratio obtained from the water solutions subjected to acid, ethanolic and calcium precipitation were 2.95 ± 0.28, 2.29 ± 0.44 and 3.10 ± 0.05 respectively. Significantly lower are the M/G values obtained in precipitates from DES solutions, indicating a higher content of L-guluronic fractions compared with precipitates from water. Among the M/G ratios obtained from each DES solution, when acid was applied the M/G from Urea and Glucose was higher than the rest. When the ethanol-water mixture was applied, higher M/G values were obtained with urea and glucose as well. On the other hand, when calcium chloride was used the highest M/G ratio was found in the precipitate from the glucose-containing DES.

4. Discussion

When alginate needs to be separated from a solution, its chemical properties could be taken as an advantage. The pK_a of mannuronic and guluronic acid are 3.38 and 3.65, and it has been reported that the polymer pK_a is in the range of 3.5 and 4.6 [6,33]. Thus, soluble forms of alginate such as its sodium salt, can be precipitated lowering the pH of the solution. In these conditions, the polymer is protonated and the macromolecule forms insoluble fibres. When acid precipitation was applied in DES solutions, the alginate recovery yields obtained were significantly lower than the recovery from a water solution. This is especially noticeable in the case of the Choline Chloride-Urea DES.

Data suggest that only a fraction of the total amount of alginate was able to be precipitated with the applied acidic conditions. This would mean that part of the alginate was either unable to interact with these conditions due to the presence of DES or due to a change in its structure making it unable to precipitate. A fraction of the dissolved alginate could have suffered a certain level of degradation reactions causing a reduction of the precipitable alginate. It is known that the solubility of alginate molecules at low pH is a function of the intrinsic viscosity that in turn is a function of the molecular weight. Low intrinsic viscosity alginates will have a short polymer chain and tend to remain dissolved at low pHs [6,34,35].

The use of choline chloride-based DES has been reported to increase the delignification of lignocellulosic material [36]. This degradation process is linked to the choline chloride compound, which cleaves certain chemical bonds between lignin and cellulose (β-O-4) and provides better conditions for further processing. It was also reported that very small proportions of choline chloride relative to the HBD used (lactic acid), are sufficient to observe this behaviour [29,36]. Considering the potential of this set of DES for degradation/-lysis/oxidation process. It is plausible that choline chloride-based DES do not discriminate among the β-O-4 found in lignin and the O-glycosidic bonds between the uronic acid units as this bond is one of the most unstable [37]. Thus, the DES together with sulfuric acid could have induced an increased lysis effect even at diluted concentrations of acid.

A process potentially occurring in parallel or even before the precipitation step, is an aminolysis process. It is described as the lysing of a molecule by reacting with primary amines [38], molecules with a lone pair. The aminolytic depolymerization of polyethene terephthalate (PET) was studied with the assistance of choline chloride-based DES as a catalyst, including choline chloride: Urea in a molar ratio of 1:2 [39]. In the previously mentioned report, the amine molecules used were diethanolamine and ethanolamine. Having the urea molecule with two lone pairs linked to its nitrogen atoms, it is very likely that it could serve as a nitrogen reagent for the aminolysis process. Even the choline chloride itself could be part of the reaction serving as a reagent and catalyst simultaneously. It is worth mentioning that if an aminolysis process occurs, the reaction products would have attached an amino group to the main molecular structure, thus, other techniques for the detection of these chemicals should be developed.

It was observed that the recovery yield was higher in the other DES solutions compared with CC-Urea DES, being the lowest of them (Ethylene glycol DES) almost 2-fold the Urea one. What could also explain the reduction of the recovery yield in the rest of DES aside from the Urea one is the occurrence of a hydrolysis process. It has been reported that the use of DES acts as a cosolvent for hydrolysis reactions in the presence of enzymes [40,41]. In the aforementioned research work, the DES made of choline chloride and propylene glycol in a molar ratio of 1:2 was used in aqueous media at a 40 % *v*/v.

It is important to bear in mind that the strength of the hydrogen bond

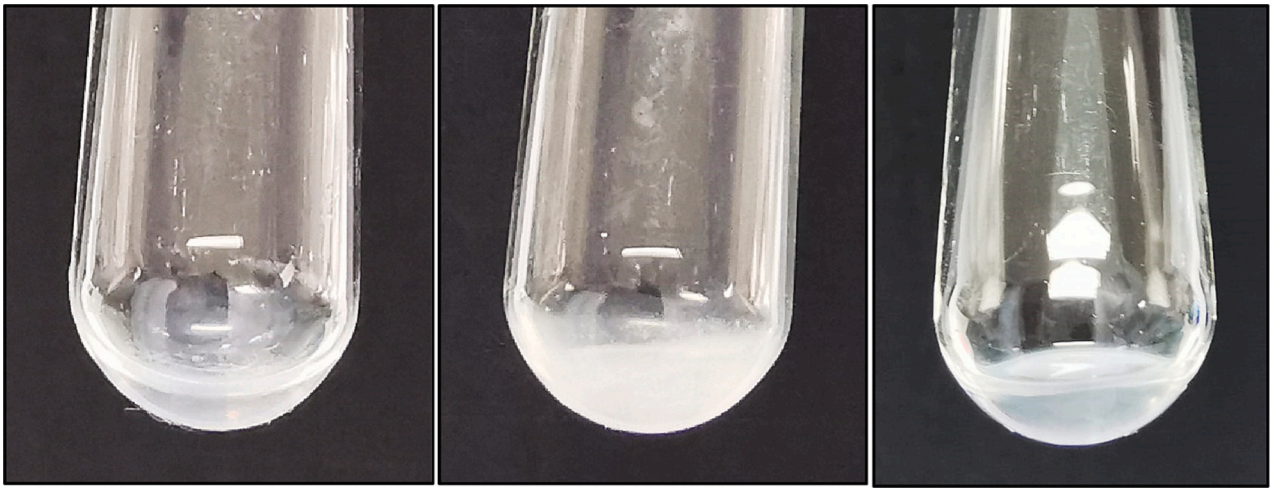

Fig. 5. Precipitate of alginate after the application of acid water (left), ethanol-water mixture (middle) and calcium chloride (right). Pictures from the DES prepared with glucose monohydrate.

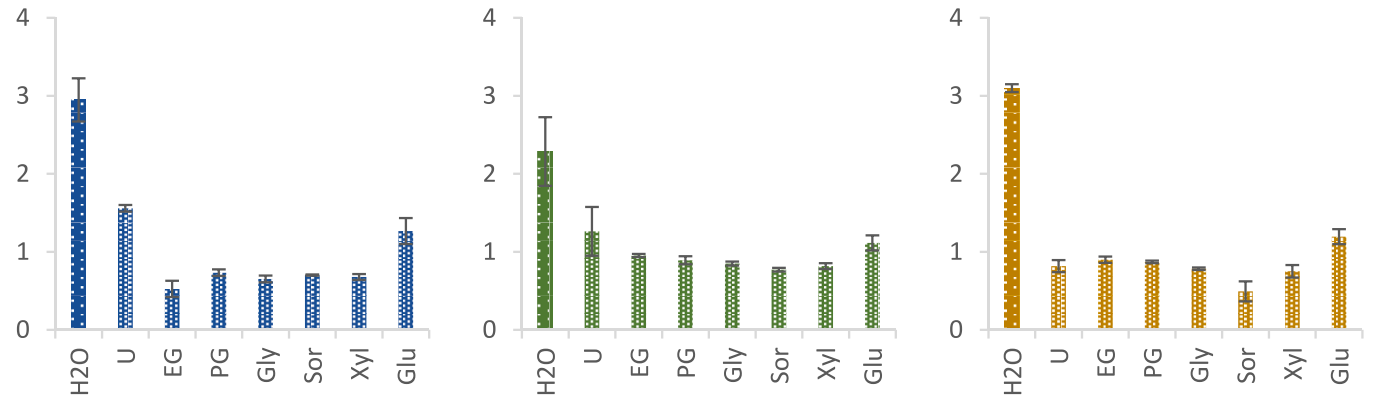

Fig. 6. M/G ratios in the alginate precipitated with acid water (left), ethanol-water mixture (middle) and calcium chloride (right) in each DES solution.

network is built among the DES compounds in which the target compounds are embedded. At such conditions, even this relatively weak non-covalent attraction could have catalytic effects if all the hydrogen bond interactions play together like a symphony [42,43]. Due to the previously described phenomena, it is plausible that shorter alginate fractions have been released. Evidence suggests that the sole presence of choline chloride-based DES might have a catalytic effect on the lysis processes of alginate. However, this reaction seems to be greatly enhanced with acidic conditions. Then, as shorter chains (or even trimers, dimers, or monomers) are less prone to precipitate even in acid conditions, a potentially degraded alginate fraction would remain in the solution reducing the alginate recovery yield.

Contrary to what is observed in the acid precipitation, higher yields were observed when the ethanol-water mixture was applied in DES solutions. When ethanol is mixed with water it forms a strong hydrogen-bond network [44]. Additionally, the solubility of alginate in ethanol is very low and consequently alginate precipitates. It has been reported that ethanol has an effect on the intrinsic viscosity of the alginate and reduces the extension of the polymer chain at 20 % (wt) ethanol, producing gels with poorly connected strands [35]. As the precipitate obtained with the ethanol-water mixture was dust-like instead of gel-like, it might imply that the gel-forming capacities of the alginate have declined. This could be explained by the effect of ethanol over the physicochemical properties of the polymer, but also due to the degrading effects that the choline chloride-DES could have had on it. Similar to what was observed in the acid precipitation although with less extent. It is important to consider that the solubility of choline chloride in ethanol is much lower [45,46]. Thus, its precipitation could occur to some extent, producing a dusty appearance.

Recovery yields obtained after the ethanolic precipitation applied in the DES solutions did not vary significantly among them. However, the recovery yield from the control solution (water) was significantly lower compared to the yields from the DES solutions (<2-fold). It is important to consider that the volume ratio of the test solutions and ethanol-water mixture (80 % *v*/v) used (1:4) dilutes the final ethanol concentration to 64 % (*v*/v) reducing its effectiveness. Nevertheless, this effect should have been also observed in the DES solutions. Interestingly this was not the case. What seems to affect this phenomenon, are the DES compounds, and potentially the presence of choline chloride as the common compound among all DES mixtures. Smidsrød & Haug [47] studied the effect of salts over the precipitation of alginate in different mixtures of water-ethanol. The authors reported that for a given ethanol concentration, a certain amount of salts is needed to precipitate the alginate. As the control solution containing alginate was prepared with milli-Q water, no salt was present, potentially reducing the effect of ethanol on the precipitation. On the other hand, it is very plausible that the choline chloride, a quaternary ammonium salt, has aided the ethanol in the precipitation process observed in DES solutions.

Divalent cations such as Ba^{2+}, Sr^{2+} and Ca^{2+}, have a high affinity with alginate polymers. This affinity is expressed as network formation among poly-α-L-guluronate fractions and the aforementioned cations and this behaviour is described by the so-called "egg-box" model. In this model, a divalent atom, such as Ca^{+} interacts with different oxygens of two adjacent guluronate units from two alginate chains [6,10]. This affinity with calcium is not observed in poly-β-D-mannuronate fractions nor alternating uronic acid blocks [6]. This alginate-calcium affinity is evident when calcium chloride is applied to the DES solutions. Except for the sorbitol and xylitol cases, the recovery yields were not significantly different to what was observed with ethanolic precipitation. However, those recovery yields are still lower than the ones obtained from a water solution. This reduction in yields could still be explained by the potential degradation of the polymer due to the catalyst effect of the choline chloride DES again. Additionally, the presence of DES compounds could be affecting the ionic strength and, with that, the intermolecular interaction of the polymer and calcium ions. Ion strength has an impact on the chain extension of the polymer [6].

Interestingly, the recovery yields observed in DES prepared with sorbitol and xylitol were much lower compared with other DES. Data suggest that despite the high concentration of calcium ions, the binding with alginate polymer was very limited. It has been reported that xylitol and sorbitol form water-soluble chelates with many bivalent and trivalent metal cations, such as Cu(II) [48–50]. Additionally, stable sorbitol-chelated calcium was prepared for agricultural applications [51]. The chelating properties of these polyols would explain the reduced capacity of calcium chloride to be applied for alginate separation. Higher concentrations of calcium solution or a higher volume ratio of DES solution and calcium solution can be used to overcome this issue.

It is worth highlighting that despite the catalytic effect that a choline chloride-based DES might have, evidence from precipitation with an ethanol-water mixture and calcium chloride also suggests that this phenomenon is only noticeably enhanced with acidic conditions. Then, choline chloride-based DES would remain as a good group of solvents if those conditions are not reached.

All precipitation methods applied in the water solution resulted in higher M/G ratios compared with the ones obtained from DES. Although particularly high, these M/G ratios fit into what was reported for some species of brown seaweed such as *Fucus guiryi* and *Laminaria ochroleuca* [15]. Sigma-Aldrich [52] indicates that the proportions of mannuronic acid and guluronic acid in their product are in the ranges of 60–70 % and 30–40 % respectively. This represents a ratio of 2.33 when proportions are 70 and 30 % for mannuronic and guluronic acid respectively. This ratio matches with what was found for ethanolic precipitation in this research work (2.29 ± 0.44). The ratios from precipitates obtained from water using acid and calcium solutions were 2.95 ± 0.28 and 3.1 ± 0.05, respectively. Despite these values out of the Sigma-Aldrich™ range, there is no significative difference among the M/G data of the different precipitation techniques. However, it is important to bear in mind that the precipitation method could influence the M/G ratio of final alginate

[15,53].

On the other hand, significantly lower were the M/G ratios obtained among the DES solutions, indicating a higher presence of polyguluronate fractions. Assuming a potential degradation effect (lysis of glycosidic bonds) of DES compounds on the alginate structure, the guluronate fractions may be less prone to be degraded. It has been stated that the reactivity of glycosidic bonds is higher in MG blocks due to their higher degrees of freedom compared with the MM and GG blocks [54,55]. This less reactivity of the GG blocks might have resulted in higher proportions of guluronate-rich fractions, which would have remained in the DES solutions for the later precipitation processes at three different conditions.

5. Conclusions and recommendations

This research work represents a significant and relevant step to develop a new process for the extraction of alginate using Deep Eutectic Solvents. In it, three precipitation methods were applied over a set of choline chloride DES for further alginate separation. Recovery yields data suggests that a better separation can be obtained using an ethanol-water mixture at 80 % *v*/v (51.2 ± 1.3 %) followed by using calcium chloride dihydrate solutions at 1 % *w/v* (45.7 ± 1.2 %), with exceptions when DES-containing polyols were used (3.6 ± 0.7 % when sorbitol DES was used). Higher concentrations of calcium solutions should be tested to avoid the potential chelation effect of the polyols. Low recovery yields (9.6 ± 1.3 %) from the precipitate using choline chloride – Urea DES in combination with acid precipitation warns of the potential role the DES might have as a catalyst for degradation of the polymer. Thus, this precipitation technique should be avoided, although it opens a potential research topic. Additionally, Lower M/G ratios in alginate precipitates from DES suggest a potential molecular interaction as well. Hence, a future assessment of the arrangement of the MM, GG and MG blocks along the polymer chain, and the molecular weight distribution in the final alginates, would provide a deeper understanding of the aforementioned interactions. Finally, other HBAs with high affinity but less potential reactivity with the phycocolloid should be screened.

In summary, this research work provides insights into the feasibility of chemical techniques for alginate separation from DES. Evidence of interactions between the solvents and the precipitation methods that could affect the separation efficiency of the polymer is offered. Despite that the previous statement conditions the selection of the separation method as a function of the DES composition, it opens the door for using these methods as pretreatment for alginate-containing DES extracts for its proper quantification.

CRediT authorship contribution statement

Wimar Reynaga-Navarro: Writing – original draft, Methodology, Investigation, Conceptualization. **René H. Wijffels:** Writing – review & editing. **Michel H.M. Eppink:** Writing – review & editing. **Antoinette Kazbar:** Writing – review & editing, Supervision, Project administration, Funding acquisition, Conceptualization.

Declaration of competing interest

The authors declare the following financial interests/personal relationships which may be considered as potential competing interests: Antoinette Kazbar reports financial support was provided by Dutch Research Council (NWO). If there are other authors, they 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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