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ORIGINAL ARTICLE

New insight for spent hops utilization: simultaneous extraction of protein and xanthohumol using deep eutectic solvents

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Abstract

Agro-food by-products are a cheap source of bioactive and functional compounds that should be used via a biorefinery approach to produce a range of bio-based products. Spent hops (SH) are an important by-product of the brewing industry and are rich in valuable ingredients such as proteins and xanthohumol (XN). Considering the increasing demand for plant-based proteins and the broad spectrum of XN bioactivity, it is important to develop eco-friendly and cost-effective techniques to extract these components. In this work, a simple, one-pot, green method for the simultaneous extraction of proteins and XN from SH using deep eutectic solvents (DESs) was developed. Protein and XN-rich precipitates were obtained by adding water (antisolvent) to the DES extracts. To determine the XN content, the precipitates were extracted with methanol and the extracts were analyzed by HPLC. The presence of protein in the methanol-insoluble fractions was confirmed by techniques such NMR, IR, elemental analysis, and SDS-PAGE. The protein content varied between 40 and 64%, which was up to 87% higher than the protein content in the SH. This study demonstrates the potential use of SH and DESs to develop an environmentally friendly method to obtain protein and XN-rich products.

Keywords Plant protein extraction · *Humulus lupulus* · Agro-food by-products · Residues valorization · Natural deep eutectic solvents (NADES) · Green extraction

1 Introduction

The sustainable management of natural resources is currently not only a challenge but also a necessity for ecological, economic, and social reasons. Therefore, raw materials should be used as efficiently as possible, and the utilization of biomass via a biorefinery approach to produce a spectrum of bio-based products and bioenergy is needed [1]. In this context, agro-food by-products are cheap sources of bioactive and/or functional compounds that can be reprocessed and utilized for the production of value-added products [2–5].

Hops (female inflorescence of *Humulus lupulus* L.) have long been used for medicinal purposes because of their sedative, antioxidant, and antimicrobial properties [6]; however, approximately 98% of the world hops production is used in the beer brewing process [7]. Hops are an essential ingredient in beer production, being responsible for the characteristic bitterness and aroma of the beverage [7, 8]. In the brewing industry, hops extracts are increasingly obtained by supercritical carbon dioxide (SC-CO_2) extraction. This extraction process is eco-friendly, the extracts obtained in this way are stable, and the subsequent brewing process is more efficient than when whole hops or pellets are used [7, 9, 10]. Although the SC-CO_2 extraction process allows for very effective isolation of the bitter acids and essential oils present in hops, polar compounds such as flavonoids and proteins are not extracted in this process and remain in the biomass residue, known as spent hops (SH) [11]. The current interest in SH is primarily the xanthohumol (XN) it contains. This prenylflavonoid has a broad spectrum of biological activities, and hops are its main natural source. XN exhibits anticarcinogenic, anti-inflammatory, antioxidant, antibacterial, antifungal, antiviral, and antiobesity activities [12–15]. It is worth noting that SC-CO_2 SH contains approximately 30% crude protein [16], which indicates that this biomass residue is relatively rich in proteins [17]. Unfortunately, because of its bitter taste, this by-product has not

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been directly applied as food or feed supplements and is mainly used as fertilizer [18–20].

Currently, there is increasing demand for plant-based proteins because of their nutritional and functional properties. Therefore, it is important to develop cost-effective and eco-friendly techniques for extracting proteins from renewable and sustainable sources [17, 21]. In recent years, proteins have also attracted increasing interest as delivery systems for bioactive compounds [22–28]. O'Connor et al. reported that SH bound to a rice protein matrix enhances circulating levels of XN after acute oral intake in humans [29].

Conventionally, plant proteins are extracted using various solvents, such as water, alkali, salt, and organic solvents. These chemical methods may also be used in combination with non-conventional cell disruptive methods (e.g., enzyme, microwave, ultrasound, high pressure, and pulse electric field) to improve the recovery of proteins [30]. However, the development of more efficient and eco-innovative technologies for plant-based protein extraction is still desirable.

Deep eutectic solvents (DESs) have been recognized as alternative green solvents for the extraction of a wide spectrum of compounds. DESs are mixtures of two or more compounds containing hydrogen bond donors (HBDs) and hydrogen bond acceptors (HBAs) in a specific molar ratio. DESs have many advantages: they are cheap, are easy to prepare, are non-volatile, biodegradable, and have low toxicity [31, 32]. The most common natural products that are efficiently extracted using DESs are phenolic compounds [33–36]. Recently, DESs have also been used for the extraction of proteins from plant materials [37–41].

Most of the published research on the use of DESs as green solvents have focused on the extraction of one specific group of compounds (e.g., phenolic compounds or proteins). A comprehensive analysis of the DES extract composition may be difficult or impossible because of the use of determination methods that are dedicated to only one group of compounds. In this context, the recovery method of the targets from DES extracts is also very important. To the best of our knowledge, studies describing the simultaneous extraction of proteins and phenolic compounds using DESs are limited to the work of Hernández-Corroto et al. [37], who described analytical methods for the extraction of these compounds from pomegranate peel.

We described a simple method for the extraction of XN from SH using DESs in our previous work [42]. XN-rich precipitates were obtained by adding water (antisolvent) to the DES extracts. To determine the XN content in the obtained precipitates, they were extracted with methanol, and then the alcohol extracts were analyzed by HPLC. It was observed that a portion of the obtained precipitates was insoluble in methanol. This work is a continuation of our previous research and focuses on the analysis of the protein-rich methanol-insoluble fractions using NMR, IR, elemental analysis, and SDS-PAGE. Herein, we present a holistic approach to the treatment of SH using DES, providing the simultaneous extraction of XN and proteins in a simple, one-pot, green method.

2 Materials and methods

2.1 Materials

SH were supplied by the New Chemical Syntheses Institute (Puławy, Poland) and were obtained from the production of hops extracts using SC-CO_2 extraction [10]. The hops cones used were (*Humulus lupulus* L.) cv. "Magnum" collected in 2015 in the Lublin region (SE Poland). Glycerol, ethylene glycol, propylene glycol, lactic acid (80 wt%), and methanol were purchased from Chempur (Piekary Śląskie, Poland). Choline chloride was purchased from Carbosynth Ltd. (Compton, UK). Ultrapure water was obtained using a Milli-Q system (Millipore, Bedford, MA, USA). XN that was used as a reference standard (NMR purity > 98%) and was isolated from SH according to a previously described procedure [43]. All other chemicals or solvents were of analytical grade and were purchased from Sigma-Aldrich/Merck KGaA (Darmstadt, Germany).

2.2 Analytical techniques

Solid-state ^{13}C CP-MAS NMR spectra were acquired using a 600 MHz Bruker Avance III HD spectrometer (Bruker Biospin GmbH, Rheinstetten, Germany) equipped with a Bruker 3.2 mm H(F)/X broadband resonance probe and a 14.1 T Ascend superconducting magnet. The CP experiments employed a 2-ms linearly ramped contact pulse, spinning rates of 12,000 ± 2 Hz, optimized recycle delays of 5 s, and 2048 scans. Chemical shifts were reported with respect to TMS and referenced using adamantane (29.5 ppm) as an external secondary reference.

ATR-IR analysis was carried out using a Thermo Scientific Nicolet iS10 spectrometer (Thermo Fisher Scientific, Waltham, MA, USA). Spectra were taken from 4000 to 520 cm^{-1} over 32 scans, with a spectral resolution of 4 cm^{-1} and a blank window for the background.

Elemental analysis (CHN) was performed using an EA-1110 elemental analyzer (CE Instruments, Milan, Italy). The samples were combusted at 1030 °C in an oxygen atmosphere, and the combustion products were analyzed using a thermal conductivity detector.

SDS-PAGE was performed on a Mini-PROTEAN Tetra Vertical Electrophoresis Cell System (Bio-Rad, Hercules, CA, USA) using 4–20% Mini-PROTEAN TGX Precast Protein Gels (10-well, 30 µL, Bio-Rad, Hercules, CA, USA) and

Tris–glycine-SDS running buffer (25 mM Tris, 192 mM glycine, 0.2% SDS). After solubilizing 0.8 mg of sample (P2 or DP) in 8 M urea (0.5 mL), the mixture was heated at 95 °C for 5 min in a thermoblock. Then, 10 µL of this solution was mixed with 10 µL of Laemmli buffer and incubated at 95 °C for 10 min in a thermoblock. A total of 10 µL of prepared samples and 2 µL of protein standard markers (SigmaMarker wide range, mol wt 6500–200,000 Da) were loaded onto the gel. The gel was run for 60 min at 120 V, stained with Coomassie Brilliant Blue R, and destained with an acetic acid/methanol/water mixture (1/4/5, v/v/v). Gel images were recorded using a GelDoc XR + System (Bio-Rad, Hercules, CA, USA).

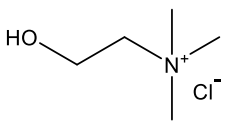
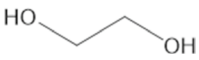
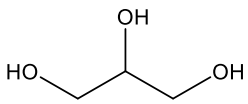
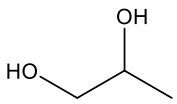
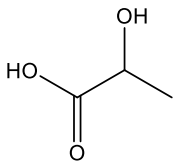
2.3 DES preparation

DESs were prepared according to a standard procedure [44]. Choline chloride as a HBA and either glycerol, ethylene glycol, propylene glycol, or lactic acid as a HBD were mixed in a 1:2 molar ratio. The mixture was stirred at 80 °C in an oil bath until a homogeneous, transparent liquid was formed (20–40 min). After cooling, an appropriate amount of deionized water (DI) was added to the resulting mixture according to our previous studies [42]. The DESs were stored at room temperature until further use. The DESs used are listed in Table 1.

2.4 Treatment of SH with DES—general method (Fig. 1)

Treatment of SH with DES was carried out according to a previously described procedure [42] with a slight modification. SH (4 g; previously sieved through a 1-mm sieve) was placed into a 250-mL screw-top glass bottle and mixed with an appropriate amount of DES (Table S1 in the Supplementary Material). The mixture was stirred (at 60 °C for 1 h in an oil bath). After cooling to room temperature, the contents of the bottle were transferred to a self-constructed extrusion press (Fig. S1 in the Supplementary Material) equipped with a 200-µm nylon mesh filter (Gwarant-Eko GEKO FILTRATION Sp. z o.o., Końskie, Poland). The bottle was rinsed with fresh DES (2 × 5 mL), and the rinsings were transferred to the press. The extruded DES extract was collected in a 400-mL

Table 1 Abbreviations and compositions of the DESs

Abbreviation	HBA[a]	HBD[b]	Molar ratio (HBA:HBD)	Water content [wt%]
DES-1	Choline chloride 	Ethylene glycol 	1:2	10
DES-2		Glycerol 	1:2	5
DES-3		Propylene glycol 	1:2	5
DES-4		Lactic acid 	1:2	5

[a]*HBA* hydrogen bond acceptor. [b]*HBD* hydrogen bond donor

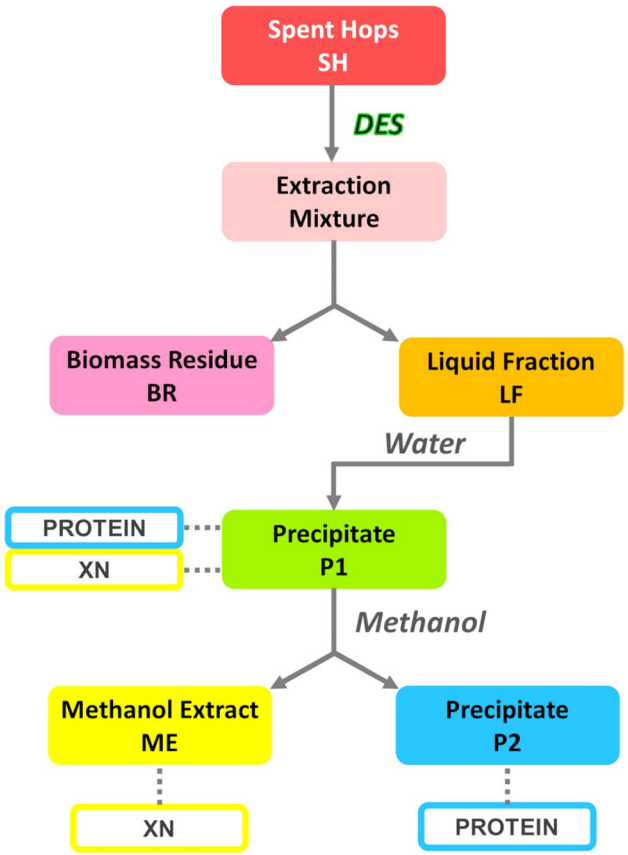


Fig. 1 Schematic representation of SH pretreatment using DES

centrifuge bottle and centrifuged at 4075 g for 30 min at 4 °C (Eppendorf Centrifuge 5810 R, Hamburg, Germany) to separate any residual fine SH particles. The clear supernatant was decanted into a glass bottle (1000 mL) and denoted as the liquid fraction (LF). For example, the LFs obtained after treatment with DES-1 and DES-2 were denoted DES-1-LF and DES-2-LF, respectively. Then, an appropriate amount of DI water (antisolvent) was added to the LF (Table S1 in the Supplementary Material), and the mixture was incubated at 4 °C for 12 h. The resulting precipitate was separated by centrifugation (30 min, 4 °C, 3220 g). The collected precipitate was transferred to a 50-mL conical centrifuge tube, washed with DI water (3 × 20 mL) to remove the remaining DES, centrifuged after each wash (30 min, 4 °C, 8819 g), and then freeze-dried (LYO GT2-Basic, SRK Systemtechnik GmbH, Riedstadt, Germany). The lyophilized precipitate was denoted as P1. P1 obtained after treatment with DES-1 and DES-2 were denoted DES-1-P1 and DES-2-P1, respectively. The biomass residue retained on the press filter was quantitatively transferred to a Büchner funnel, washed with DI water (3 × 50 mL), vacuum-filtered, and then freeze-dried. The lyophilized biomass residue was denoted as BR. For example, BR obtained after treatment with DES-1 and DES-2 were denoted DES-1-BR and DES-2-BR, respectively. Each experiment was performed in triplicate.

2.5 Extraction of XN from precipitate P1

The extraction and determination of the XN content in P1 were carried out according to a previously described method [42]. Briefly, 40 mg of P1 and 20 mL of methanol were placed into a conical centrifuge tube (50 mL). The mixture was vortexed (1 min) and then centrifuged (30 min, 4 °C, 8819 g). The supernatant (methanol extract) was transferred to a 100-mL volumetric flask, and the remaining (methanol-insoluble) precipitate was re-extracted with methanol (2 × 10 mL). The mixture was vortexed and centrifuged after each extraction, and the supernatant was transferred to the same volumetric flask. After filling the volumetric flask with methanol, the solution, denoted as ME, was analyzed by HPLC. Subsequently, the methanol was removed from the remaining alcohol-insoluble precipitate by vacuum evaporation at 60 °C, and the resulting dry precipitate was denoted as P2. For example, P2 samples obtained after treatment with DES-1 and DES-2 were denoted DES-1-P2 and DES-2-P2, respectively. Each experiment was performed in triplicate.

2.6 Extraction of protein from SH using TCA/acetone/phenol

The protocol described by Wang et al. [45] was used to extract proteins from the SH for SDS-PAGE analysis. Before extraction, the SH were ground into a powder in liquid nitrogen using a mortar and pestle. The powdered SH (100 mg) was transferred to a 2-mL tube. The tubes were then filled with 10% trichloroacetic acid (TCA) in acetone, vortexed, and centrifuged at 20,817 g for 5 min at 4 °C. The supernatant was removed by careful decanting. The pellet was re-suspended in 0.1 M ammonium acetate in 80% methanol, vortexed, and centrifuged (5 min, 4 °C, 20,817 g). After discarding the supernatant, the pellet was re-suspended in 80% acetone, vortexed, and centrifuged at 20,817 g for 5 min at 4 °C. The supernatant was discarded by careful decanting, and the pellet was air-dried to remove residual acetone. The air-dried pellet was re-suspended in 0.4 mL of Tris-buffered phenol (pH 8.0) and 0.4 mL SDS buffer (30% sucrose, 2% SDS, 0.1 M Tris–HCl, pH 8.0, 5% 2-mercapthoethanol), mixed thoroughly, incubated for 5 min at room temperature, and centrifuged at 20,817 *g* for 5 min at 4 °C. The upper phenol phase was transferred to a new 2-mL tube, and the tube was filled with 80% methanol containing 0.1 M ammonium acetate and stored at − 20 °C overnight. The mixture was then centrifuged at 20,817 g for 5 min at 4 °C. The supernatant was discarded, and the pellet was washed with methanol and then with 80% acetone. During each washing, the

pellet was vortexed and centrifuged (5 min, 4 °C, 20,817 g). Finally, the pellet was air-dried, and the resulting dry pellet was denoted as DP.

3 Results and discussion

3.1 Pretreatment of SH with DES

In this study, four choline chloride-based DESs containing ethylene glycol (DES-1), propylene glycol (DES-2), glycerol (DES-3), or lactic acid (DES-4) as the HBD were used for pretreatment of the SH. The motivation to use such DES components was their wide availability, low cost, and biodegradability [46, 47]. It should be highlighted that these DES components except ethylene glycol, have GRAS status. Among the tested alcohol-based DESs, as HBDs were selected compounds differing in alkyl chain length (C2 or C3), and the number of hydroxyl groups (2 or 3). For comparison, DES containing lactic acid (C3, one hydroxyl group, and one carboxyl group) as HBD was also used. The DES composition and extraction conditions were selected based on our previous research (Table 1 and Table S1 in the Supplementary Material) [42]. As shown in Fig. 2, the mass loss of the SH after DES treatment varied between 32 and 45%. The smallest mass loss was observed for DES-2 (32.3%).

3.2 Determination of XN content in precipitate P1

Precipitates P1 were obtained by adding water (antisolvent) to the liquid fraction (LF). As shown in Fig. 3, the highest P1 yields were obtained using DES-4 and DES-1 (2.98 and 2.83 g/100 g SH, respectively). Slightly lower yields were obtained using DES-3 and DES-2 (2.20 and 2.11 g/100 g SH, respectively).

To determine the XN content in P1, the precipitates were extracted with methanol and then the alcoholic extracts were analyzed by HPLC. Figure 4 shows the results. The highest

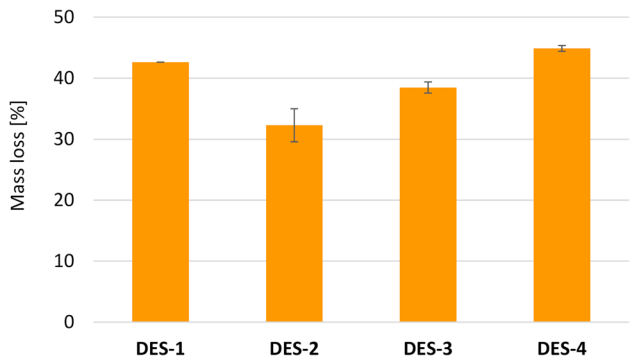


Fig. 2 Mass loss of SH after DES treatment

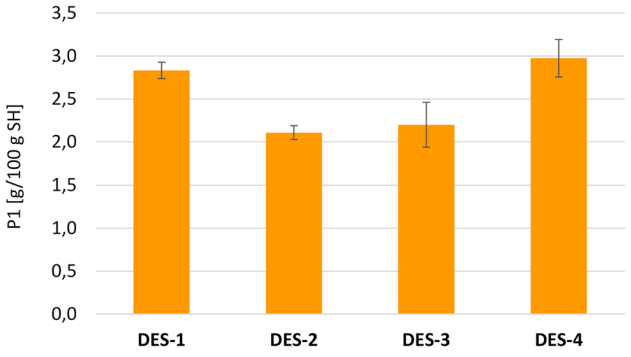


Fig. 3 Yield of precipitate P1

XN extraction yield (1.92 mg/g SH) was obtained using DES-4, whereas DES-3 was the least effective (0.52 mg/g SH).

It is well known that the extraction efficiency of the target compounds is affected by many factors, such as pretreatment time, extraction temperature, DESs type, biomass/DES ratio, water content in DESs, and HBA/HBD molar ratio. The structure of a DES determines its physicochemical properties, including viscosity, density, polarity, pH, and surface tension, and is crucial in determining extraction efficiency [34, 48, 49]. Although many physicochemical properties of the solvents used in our work are known [50–57], the determination of a clear rule to define their extraction efficiencies remains limited. A valuable example showing the complex relationship between the DES formulation and extraction efficiency are studies on the delignification of lignocellulosic biomass. Despite numerous studies on this subject, there is no comprehensive understanding of the correlation between the lignin extraction efficiency and the structure as well as properties of DESs [58, 59].

Compared with the results obtained in our previous work [42], the XN extraction yield obtained herein was 19% higher using DES-4, but 25%, 39%, and 77% lower using DES-1, DES-2, and DES-3, respectively (Fig. S2). These differences may be a result of the scale-up of the process

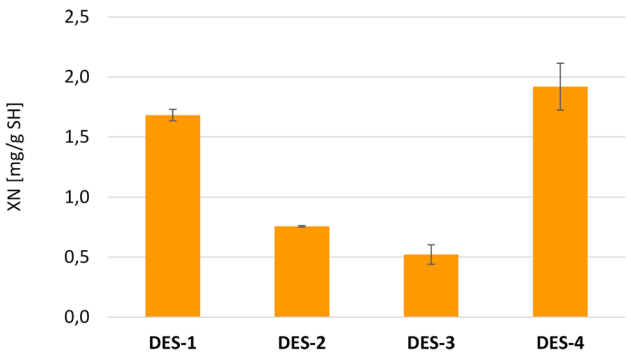


Fig. 4 Effect of various DESs on the amount of XN extracted from P1

(eightfold compared with the previous experiments) causing the formation of precipitates with other particle sizes. In the case of DES-3, these differences were clearly visible to the naked eye. When carrying out the process on a smaller scale (0.5 g SH), the formation of large aggregates was observed, whereas when using 4 g SH (maintaining the other extraction parameters the same), a precipitate of very small particles formed. The separation (centrifugation) conditions used in this study may have been insufficient to effectively separate the precipitates obtained. We speculate that a different separation method could be used to solve this problem (e.g., ultrafiltration and diafiltration). It is known from the literature that various parameters, such as compound concentration, solvent to antisolvent volume ratio, stirring speed, and temperature, strongly influence the particle formation mechanism and determine the particle size and distribution [60–62]. The above observations indicate that the antisolvent precipitation process is a critical step that must be individually selected for each DES. Particular attention should be paid to this step, especially when applying this method on a large scale.

Compared with conventional XN extraction methods employing volatile organic solvents, the method we developed is very simple, green and selective. Although higher or similar XN extraction yields can be obtained using organic solvents, these methods are not selective. To separate XN from the remaining components of the extract, multistep purification methods which usually involve chromatography, precipitation, and crystallization are required [8, 43, 63]. These steps significantly reduce the isolated yield of XN, are time-consuming, and require the use of large amounts of volatile organic solvents, which are often flammable and toxic. For example, Anioł et al. [63] isolated 2.04 g of XN from 1 kg of SH by the extraction with acetone, purification on silica gel (using mixture of chloroform and methanol as eluent), and subsequent crystallization from methylene chloride. Stevans et al. [8] isolated 445 mg pure XN from 500 g hops (which is < 1 mg/g). However, several solvents and two chromatographic columns were required. It should be emphasized that the comparison of the XN extraction efficiency is difficult because it depends on many factors: hops variety, growing conditions, climatic factors, harvest time, etc.

3.3 Analysis of precipitate P2

Only part of P1 dissolved in methanol during the XN extraction. The alcohol-insoluble precipitates, P2, were dried and weighed. As with P1, the highest P2 yields were obtained using DES-1 and DES-4: 2.24 and 2.13 g/100 g SH, respectively (Fig. 5). Using DES-2 and DES-3, similar lower amounts of P2 were obtained (1.56 and 1.51 g/100 g SH, respectively).

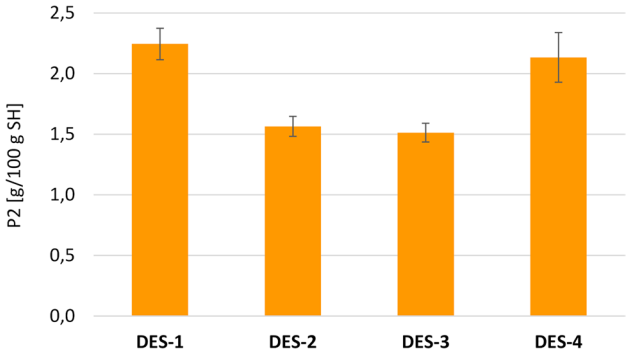


Fig. 5 Yield of precipitate P2

Note that the obtained precipitates differed in color (Fig. 6). P1 precipitates have a yellow hue owing to the presence of XN (Fig. 6a). Among the P2 precipitates, DES-1-P2 had the most intense dark color, whereas DES-3-P2 had the lightest color (Fig. 6b). The dark color may be due to the presence of tannins. According to the literature, the tannin content in hops is 3–6% [64, 65]. Abbot et al. (2015) reported that vegetable tanning agents have good solubility in the DES ethylene glycol:choline chloride [66]. It is well known that tannins have the ability to form complexes with proteins. Tannins efficiently precipitate proteins at a pH near their isoelectric point [67, 68]. However, tannin-protein complexes can also form at a pH far from the protein

Fig. 6 Samples of precipitates P1 (**a**) and P2 (**b**)

isoelectric point [69]. The pH of the solutions obtained after adding antisolvent (water) to LF were 5.1, 5.3, 4.9, and 1.5 for DES-1, DES-2, DES-3, and DES-4, respectively. The low pH of an aqueous solution of DES-4-LF is due to the presence of lactic acid as the HBD in this solvent. The obtained data indicate that there is no strict correlation between the pH of the solutions and the amount and color of the precipitates obtained.

3.3.1 Solid-State ^{13}C CP-MAS NMR analysis

Figure 7 shows a comparison of the ^{13}C CP-MAS NMR spectra of P2, SH, and BR. The spectra show the broad signal of a carbonyl group between 179 and 168 ppm, which can originate from proteins as well as from hemicellulose, pectin, and lignin [70, 71]. The intensity of this signal was significantly higher in the P2 spectra than in the SH and BR spectra. Signals in the 160–114 ppm region in the P2 spectra indicate the presence of aromatic carbons, which may originate from the side chains of amino acids as well as from lignin and tannins [70, 71]. The signal at 105 ppm is attributed to the C1 hemiacetallic carbons of cellulose. The spectra also show characteristic signals from polysaccharide carbons at 89–83 (C4), 77–70 (C2,3,5), and 66–61 (C6) ppm [71, 72]. Compared with those in the spectra of the SH, the carbohydrate carbon signals in the spectra of P2 were drastically decreased. The exception was DES-3-P2, in which the signals from the polysaccharide carbons were more evident. Noteworthy is the presence of a broad signal between 60–48 ppm in the P2 spectra, which can be assigned to the α-carbons of proteins [70, 73]. This signal is not visible in the BR spectra. The signal at low chemical shifts (42–10 ppm) can be attributed to the carbons of the amino acid side chains and aliphatic carbons of lignin [70, 74]. The intensity of these signals in the P2 spectra was greater than that in the spectra of the SH and BR. From the NMR data, it can be concluded that precipitates P2 are enriched in proteinaceous matter, whereas most of the polysaccharides are not extracted by DES and remain in the BR. Considering the selectivity of the extraction, BR could be considered as a source of polysaccharides. Conversion of carbohydrates can afford high value-added chemicals. Moreover, BR can also be a potential sustainable energy source.

3.3.2 Infrared (ATR-FTIR) analysis

The ATR-FTIR spectra of P2, SH, and BR are shown in Fig. 8. The spectra of precipitates P2 show strong absorption bands at 1625 and 1500 cm^{-1}, which can be assigned to the amide I and II bands, respectively [75]. These bands were more prominent in the spectra of P2 than in SH and BR. In comparison with the SH and P2 spectra, the BR spectra show an increase in the intensity of the bands in the 1000–1200 cm^{-1} range, characteristic of carbohydrates [76]. The increased intensity of the amide bands and the decreased intensity of the bands characteristic of polysaccharides were the least visible in the DES-3-P2 spectrum. The increased content of polysaccharides in DES-3-P2 compared with other P2 precipitates is in agreement with the NMR data discussed in Sect. 3.3.1. The absorption band at ~ 1740 cm^{-1}, visible in the SH and BR spectra, corresponds to a carbonyl group, which can be derived from hemicellulose, pectins, or lignins [76, 77]. The intensity of this band decreased or disappeared in the P2 spectra.

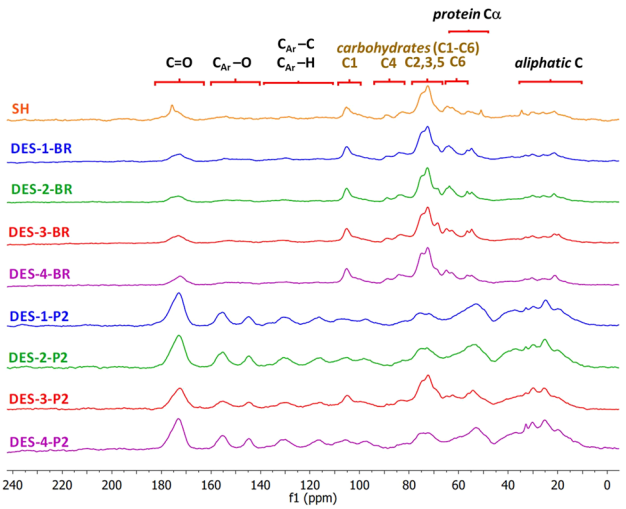


Fig. 7 ^{13}C CP-MAS NMR spectra of the SH, BR, and P2 samples

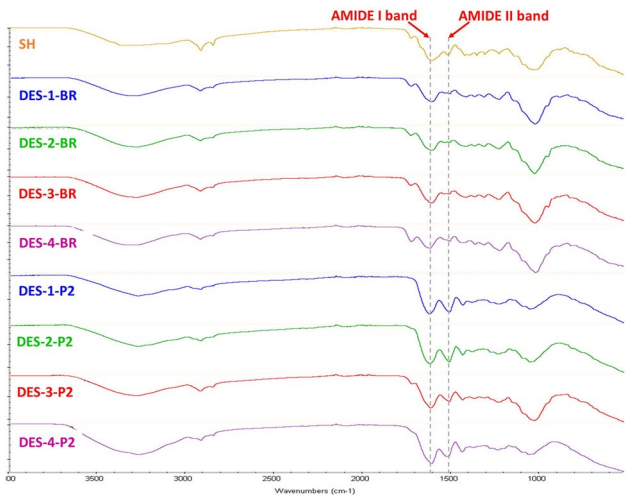


Fig. 8 ATR-FTIR spectra of the SH, BR, and P2 samples

3.3.3 Elemental analysis

Elemental analysis (CHN) was performed for the SH, BR, and P2 samples (Fig. S3). The protein content in the samples was calculated from the nitrogen content using a conversion factor of 6.25. Depending on the DES used, the protein content in P2 varied between 40 and 64% (Fig. 9). It is worth noting that the protein amounts in DES-1-P2 and DES-2-P2 were 87% and 74% higher, respectively, than in the SH. The protein content of all BRs was comparable (19–21%). This confirms our assumptions described in Sect. 3.2 that part of the P1 precipitates (especially in the case of DES-3) were not effectively separated from the solution obtained after adding water to the LF.

To the best of our knowledge, the literature describes methods for extracting hops proteins for analytical purposes only. For example, Neugrodda et al. developed an extraction method for the Lab-on-a-Chip technology that can be used for the characterization of hops varieties based on their protein profile [78].

3.3.4 Electrophoretic (SDS-PAGE) analysis

SDS-PAGE analysis was performed for precipitates P2 and the pellets (DP) obtained from the SH using the TCA/acetone/phenol extraction method, and the analysis confirmed that DESs have the ability to extract proteins from SH. All samples showed characteristic polypeptide bands in the range of ~19–22 kDa and ~29–35 kDa (Fig. 10). These bands were also observed in some varieties of hops [78]. As reported by Neugrodda et al., the hops protein composition seems to be influenced by the variety of the hops [78]. There is limited information about hops proteins in the literature [78], so identification of the proteins in P2 (DES-1-P2) from SDS-PAGE gels according to the bottom-up strategy using the nano-LC–MS/MS technique was carried out (Fig. S4, see the "Supplementary information" for details). During analysis of the obtained proteomic data, one should be aware that because the SH were obtained via SC-CO_2 extraction, various protein modifications might have been introduced [79, 80]. Therefore, straightforward identification or annotation of identified proteins may lead to false positive hits, despite the high Mascot or Sequest HT score and sequence coverage.

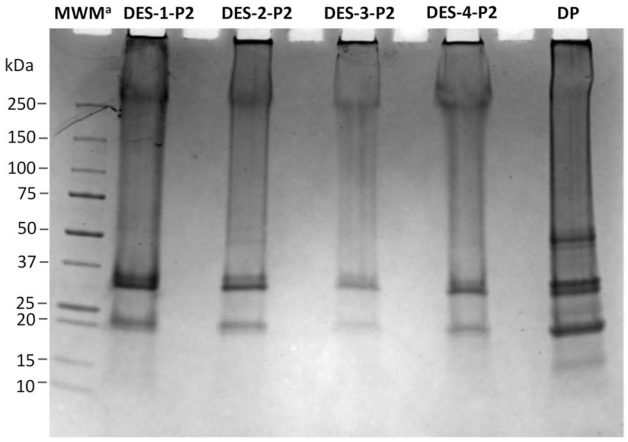


Fig. 10 SDS-PAGE patterns of P2 and DP samples. [a]MWM = molecular weight markers

4 Conclusions

This study demonstrated a simple, one-pot, green method for the simultaneous extraction of XN and proteins from SH using choline chloride-based DESs. The XN extraction efficiency was 0.52–1.92 mg/g SH depending on the DES used. The protein content in the methanol-insoluble fractions (P2) varied between 40 and 64%, which was up to 87% higher than that in the SH feedstock. Spectroscopic data (NMR and IR) indicated that most of the polysaccharides

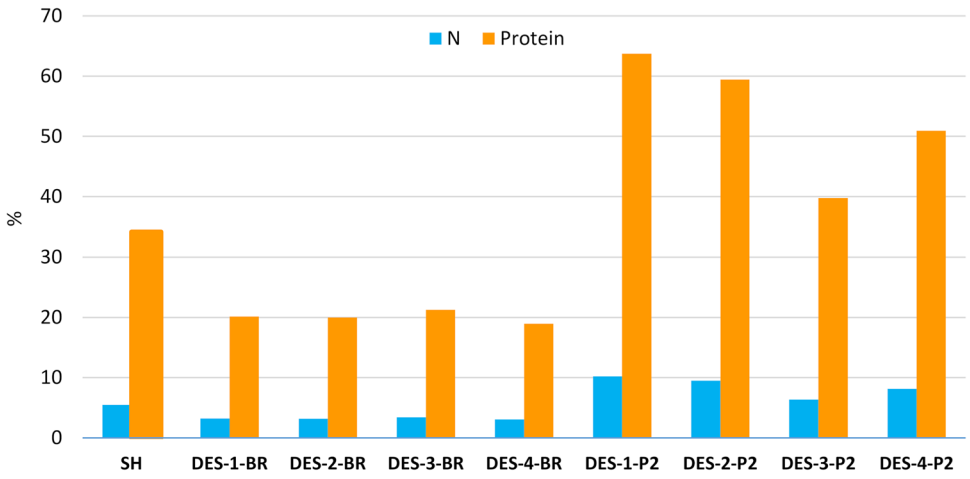


Fig. 9 Nitrogen (N) and protein contents of the SH, BR, and P2 samples

were not extracted by DES and remained in the BR. Therefore, BR can be used as a potential fermentation feedstock for biofuel and value-added chemical production. However, the appropriate studies should be conducted to confirm this hypothesis.

Owing to the broad spectrum of bioactivity, poor water solubility, and low bioavailability of XN, the development of new alternative XN delivery systems is of significant interest [29, 81]. A comparison of the biological activity and bioaccessibility/bioavailability of free XN, XN and protein-rich precipitates (P1), as well as the DES extracts (LF) would provide valuable information. We plan to conduct such research in the future. It should be noted that there is little information about the proteins in hops [78], so it is necessary to examine the nutritional and functional properties of the proteins extracted in this study.

In the developed method, green solvents were used: DESs and water (as an antisolvent). It should be emphasized that DESs have many advantages, e.g., they are easy to prepare, inexpensive, and biodegradable. However, the high viscosity is the main disadvantage of these solvents, which may limit their large-scale use [82–84]. Conceptually and prospectively, because of the presence of both XN and proteins, precipitate P1 could be considered as a "dual-function" product with the potential to be used as nutraceuticals and functional food ingredients. However, deeper studies of the precipitates obtained are needed, including the potential toxicity of the DES residuals and determination of any undesirable compounds, to gain insight into the possibilities of their use for food applications. We hope that this work will contribute to a deeper and more comprehensive analysis of the products obtained during the treatment of biomass with DESs.

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Author contributions Aleksandra Grudniewska conceived and designed the experiments. Aleksandra Grudniewska and Natalia Pastyrczyk carried out the experiments and analyzed the data. Aleksandra Grudniewska wrote the paper.

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Data availability All data generated or analyzed during this study are included in this article and its supplementary information files.

Declarations

Ethical approval Not applicable.

Competing interests The authors declare no competing interests.

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