Enhanced Protein Extraction from Oilseed Cakes using Glycerol-Choline Chloride Deep Eutectic Solvents: A Biorefinery Approach

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**ABSTRACT:** The enhanced extraction of proteins from rapeseed cake (RC) and evening primrose cake (EC) using a glycerol-choline chloride deep eutectic solvent (Glyceline) is reported. Protein-rich precipitates were obtained by adding water (antisolvent) to the DES extract derived at different processing temperatures. The presence of proteins in precipitates has been confirmed by several techniques, such as: NMR, ATR-IR, TGA, CHN and SDS-PAGE. Yield of precipitates improved with increasing temperature of treatment, reaching a maximum of 20% and 35% at 140 °C from RC and EC, respectively. In general, the protein content of the extracts was *ca.* 40–50%, which is up to 20% more than the starting materials. SDS-PAGE confirmed that glyceline selectively extracted cruciferin proteins (*ca.* 16–33 kDa) from RC, while proteins with variable molecular weight (10–40 kDa) were identified in EC extracts. As potential application cruciferin-rich RCP60 and RCP100 could be incorporated into final food formulations as a source of protein due to its light colour.

**INTRODUCTION**

Vegetable oil has one of the highest trade shares (42%) of production of all agricultural commodities. During 2014-16, average annual global production of vegetable oil was 179 Mt and is expected to increase to 220 Mt in 2026.1 The three most important vegetable oils are palm oil (33%), soybean oil (26%) and rapeseed oil (15%), representing 74% of world output. In the European Union (EU), rapeseed oil is the main vegetable oil used to make biodiesel (48% in 2016) and the EU is the most important biodiesel producer accounting for almost 37% (12.61 Mt in 2016) of global output.2

During the pressing of oilseed, in addition to the main product – oil, about 65% of by-product is formed as a cake.3 The remaining oil in the cake is usually extracted with *n*-hexane, generating extraction meal as a by-product. Currently, according to the biorefinery concept, oilseeds are used for the production of biodiesel (bioenergy), and as bio-based products the cake and glycerin are obtained.4 Often, oilseed cake or meal is used in animal feed because it has a high protein content.

The substitution of animal proteins through vegetable proteins in human diet becomes increasingly important due to the growing world population, ecological and economic aspects.5 Rapeseed meal is the second largest protein feed produced in the world after soybean. In 2016/17, world production of rapeseed meal was 35 Mt, having a global protein content around 12 Mt.2 Rapeseed protein represents an interesting opportunity as a high quality, sustainable human protein source. The predominant storage proteins found in rapeseed are cruciferin (globulin, 11S, 300-350 kDa) and napin (albumin, 2S, 12-16 kDa), constituting 80% of the total protein content of mature seeds (60% cruciferin and 20% napin, respectively). Rapeseed also contains the structural proteins such as oil body proteins (mainly oleosins, 2-8% of the total seed proteins, 18-25 kDa) and lipid transfer proteins.6–8 Rapeseed protein contains a well-balanced amino acid composition and is recognized as a high quality protein, comparable with milk and egg proteins.6,7 The limiting factors of the use of rapeseed cake (RC) as protein source in the food applications is the content of undesirable compounds, such as glucosinolates, phytates, and phenolic compounds, which are responsible for the unfavorable colors and tastes of rapeseed proteins.6,7 Most phenolic compounds identified in RC are free and esterified phenolic acids (mainly sinapine, about 1%) and condensed tannins (up to 3%),7,9 which may be extracted under alkaline conditions but then furnish a dark color in the rapeseed protein products.10

Interestingly, evening primrose (*Oenothera bienn*is L.) oil has attracted interest due to its high content of γ-linolenic acid (6.9-12.6%).11 It is used as a nutritional and a medicinal supplement to treat a variety of ailments including rheumatic and arthritic conditions, eczema, psoriasis, premenstrual and menopausal syndrome, and diabetic neuropathy.12 China is the world's largest evening primrose producer, but with the growing demand for evening primrose oil, there has been an increase in raw material cultivation in Europe and North America as well.13 Evening primrose protein is rich in sulfur amino acids, but is poor in lysine.14–18 The use of evening primrose cake (EC) as animal feed is limited due to low values of protein degradability in the rumen and intestinal digestibility. The high level of tannins (12-14%) and dietary fiber (22-23%) in EC has a negative effect on its bioavailability.14,16,17 The chemical composition of rape and evening primrose seed and pressed cake is shown in Table S1.

Deep Eutectic Solvents (DESs) have been applied as extraction solvents for flavonoids,19,20 phenolic acids,21–23 tocols,24 alkaloids,25 anthocyanins26 and other bioactive compounds from plant materials.27 Das *et al.* (2016) reported the selective extraction of κ-carrageenan from seaweed using DES.28 Recently, the extraction of proteins by aqueous two-phase system (ATPS) based on DES has been reported by several researchers.29–32 Choline chloride-based DESs were applied for selective extraction of collagen peptides from cod skins33 and for deconstructing wool for a ‘top down’ fabrication of keratin.34 Eutectic mixtures were also used for the delignification of different lignocellulosic biomass.35–38

Thus, the main aim of our research is to explore the effective use of by-products (oil cake and glycerin) obtained during the processing of oilseeds and the inclusion of choline chloride in this process – a commonly used and low-cost feed additive (Figure 1). To the best of our knowledge, Glyceline pretreatment of the oil cakes has not been studied so far. DES prepared from choline chloride and glycerol will be used for pre-treatment of cakes pressed at three different temperatures: 60, 100 and 140 °C (Figure 2). The motivation to use such DES components was their availability, low cost and that they are ‘generally recognized as safe’ (GRAS).39 Choline chloride is the naturally occurring salt of choline, which is a precursor for acetylcholine, phospholipids, and is also an important source of labile methyl groups. Choline chloride is widely used as an animal feed supplement and according to the European Food Safety Authority (EFSA) belongs to the functional group "vitamins, pro-vitamins and chemically well-defined substances having similar effect".40 Glycerol occurs naturally in lipids (*e*.*g*. triglycerides, phospholipids) and is an endogenous metabolite in mammals. It is used both for the production of cosmetics and as a food additive.41 An important factor in the selection of glycerol as the hydrogen bond donor (HBD) was the fact that it is a by-product (10%) of biodiesel production, which demand is constantly increasing.42

In the literature, several methods have been reported to recover the extracted compounds from DES, such as precipitation by addition of antisolvents,19,38,43 solid-liquid extraction (SLE) using resin,20,44 solid-phase extraction (SPE) on a C18 cartridge19 or liquid-liquid extraction (LLE) using another solvent.45 In the present study, water as an antisolvent is used. In this work we focus on the analysis of the precipitate obtained. For this purpose, several techniques were used (NMR, ATR-IR, TGA, CHN and SDS-PAGE).

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**Figure 1.** Holistic approach used to produce protein-rich extracts from oilseed cakes by means of DES.

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**Figure 2**. Schematic showing the treatment of RC and EC with DES and precipitate formation at different temperatures.

**EXPERIMENTAL SECTION**

**Materials**

Rapeseed cake (RC) and evening primrose cake (EC) pellets were obtained from Oleofarm Sp. z o. o. (Wrocław, Poland). Choline chloride (≥98.0%) was purchased from Sigma Aldrich (Poznań, Poland). Glycerin (≥99.5%) was purchased from Chempur (Piekary Śląskie, Poland). Ultra-pure water was obtained from a Milli-Q system (Millipore, Bedford, MA, USA). DES (Glyceline) was prepared according to previously described method.46 Choline chloride and glycerol were mixed in a 250-mL flask in a molar ratio of 1:2. The mixture was stirred (500 rpm) at 80 °C in an oil bath until a homogeneous, transparent liquid was formed (45 min). After cooling, the DES (Glyceline) was stored at room temperature.

**Treatment of RC and EC with DES – general method (Figure 2)**

Prior to DES treatment, RC and EC pellets were ground into fine powder using an IKA A11 grinder (IKA-Werke GmbH, Germany). The ground sample (5 g) was placed into 100 mL screw-top glass bottle and was mixed with DES (45 g) giving a biomass: DES ratio of 1 : 9 (w/w). The mixture was stirred (500 rpm) at three different temperatures: 60, 100 and 140 °C for 2 h in an oil bath. After cooling to room temperature, the content of the bottle was centrifuged for 10 min at 3500 rpm (MPW-350-RH, MPW Med. Instruments, Warsaw, Poland). The liquid fraction (supernatant) was decanted into an Erlenmeyer flask (300 mL), whilst the pellet was treated with fresh DES (3 x 5 g), re-centrifuged (10 min, 3500 rpm) and the obtained supernatant was each time transferred to the same Erlenmeyer flask with liquid fraction. The combined liquid fractions were denoted LF. For example, LF obtained after RC and EC treatment are coded RCLF and ECLF, respectively. The DES extracted pellet, now termed the biomass residue (BR) was washed with DI water (3 × 20 mL), filtered *in vacuo*, dried (105 °C, 16 h), cooled in a desiccator and weighed to the nearest 0.001 g. BR of RC and EC were denoted RCBR and ECBR, respectively. The numbers (60, 100, 140) following the acronyms RCLF, ECLF, RCBR and ECBR represent the extraction temperature in which biomass was treated. For example, RCLF60 refers to rapeseed cake liquid fraction processed at 60 °C. Each experiment was performed in triplicate.

**Precipitation of LF – general method**

To the LF obtained after treatment of the oil cakes with DES, a further 250 mL of DI water was added, and the mixture was incubated at 4 °C for 12 h. The ensuing precipitate was separated by centrifugation (10 min, 6500 rpm); the gathered precipitate was washed with DI water (3 × 20 mL), each time being centrifuged (10 min, 6500 rpm). Effective separation of the precipitate obtained after adding water to RCLF was difficult by means of centrifugation. In this case, filtration using a P3 glass crucible was used; the precipitate was washed three times with DI water (3 × 20 mL). The resulting precipitates were then freeze-dried (FreeZone Freeze Dry Systems 18L, Labconco, Kansas City, USA) and denoted as RCP and ECP based on their origin. The numbers (60, 100, 140) following the acronyms RCP and ECP represent the extraction temperature in which biomass was treated. Each experiment was performed in triplicate.

**Instrumental analysis**

For compositional analysis and physicochemical characterization of RCP, ECP, as well as RCBR, ECBR, RC and EC, several techniques were used, such as Solid State 13C CP-MAS NMR, ATR-IR, CHN and SDS-PAGE. Full instrument details are given in the Supporting Information.

**RESULTS AND DISCUSSION**

**Pretreatment of RC and EC with DES**

The mass losses of RC and EC after DES treatment are presented in Figure S1, which shows that mass losses after DES treatment increased with increasing temperature. However, during the extraction at 60 °C and 100 °C the mass losses of RC were significantly higher than in the case of EC (at 60°C: 47% and 26%, at 100 °C: 53% and 33% for RC and EC, respectively). The largest mass decrease for both oil cakes was observed at 140 °C, 57% and 52% for RC and EC, respectively.

As shown in the Figure 3 the yield of precipitate formed after adding water (antisolvent) to the LF increased with increasing the extraction temperatures. The change of the extraction temperature from 60 °C to 140 °C results in a nearly two-fold increase in the resulting precipitate in the case of RC (11.5 and 19.9 g/100 g RC, respectively), and over four-fold in the case of EC (8.4 and 34.2 g/100 g EC, respectively).

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**Figure 3**. Yield of precipitate formed after adding water to RCLF and ECLF.

It is worth noting that, RCP60 and RCP100 have a light beige color while RCP140 has a dark brown colour (Figure S2d). As shown in Figure S2e, biomass residue obtained after treatment at 140 °C (RCBR140) also has a brown color, while colour of RCBR60 and RCBR100 is similar to the RC (green-black) (Figure S2a). These changes are probably the result of reactions occurring during high temperature (non-enzymatic browning), such as Maillard’s reaction. All precipitates obtained from evening primrose (ECP60, ECP100 and ECP140) as well as biomass residue (ECBR, ECBR100 and ECBR140) have a brown color, close to the starting feedstock (EC) (Figures S2a, d, e).

It is well known that proanthocyanidin (PA, also known as condensed tannin) is frequently responsible for pigmentation in mature plant tissues, particularly in the seed coat. During seed maturation the initially colourless PA undergoes secondary changes causes seed coat darkening. These changes are the result of the formation of oxidised complexes of PA with the cell wall, polysaccharides and other phenolics in the seed coat.47

**Analysis of Products Obtained After DES Pretreatment**

**Solid State 13C CP-MAS NMR Analysis.** 13C CP-MAS spectra of the oil cakes (RC and EC), biomass residues (RCBRs and ECBRs) and precipitates (RCP and ECP) are shown in Figure 4.

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**Figure 4.** Solid State 13C CPMAS NMR spectra of RC, RCBRs, RCPs (a) and EC, ECBRs, ECPs (b) samples.

The observed 13C chemical shifts together with their assignment of peaks are summarized in Table S2. The spectra for the oil cakes (RC and EC) and biomass residues (RCBRs and ECBRs) show characteristic signals for cellulose and other structural polysaccharides (hemicellulose, pectin) carbons at 105 (C1), 89-84 (C4), 76-70 (C2,3,5) and 66-61 (C6) ppm.48-51 The spectra also show a broad signal of the carbonyl group (179-172 ppm), which can be derived from protein, pectinaceous matter, hemicellulose and lignin.50-52 Compared to the oil cakes, in the spectra of BRs the carbonyl signal intensity decreases and the signals characteristic for cellulosic matter increases. These differences are more evident in the case of evening primrose (Figure 4b), where it is also observed that the intensity of the C=O signal decreases with the increasing extraction temperature.

In the precipitates (RCPs and ECPs), while the intensity of the carbonyl group (179-172 ppm) increased, cellulose C1 signal at 105 ppm disappeared (ECPs) or was drastically reduced (RCPs). Similarly, the intensities of signals assigned to C2, C4, C5, and C6 of structural polysaccharides sugar units decreased compared to the corresponding spectra of oil cakes. The signal at 160-112 ppm in the spectra of precipitates indicates the presence of aromatic carbons, which may come from aromatic side chains of amino acids and from lignin.50-52 Aromatic C signals are more evident in ECPs than RCPs. The carbon signals recorded between 86 ppm and 72 ppm can be assigned to Cα/β-OR of lignin.53,54 In RCPs and ECPs spectra signals at 65-48 ppm can be assigned to α-carbons in protein and at 56-54 ppm51,55,56 to CH3O in lignin.50-54 The carbon signals recorded at 32-10 ppm are associated to the alkyl groups of the side chains of amino acids residues and aliphatic carbons in lignin.50-53 It is worth noting that the intensity of these signals in the RCPs and ECPs spectra is greater than in the case of BRs and oil cakes. No significant differences were observed in the spectra of the precipitates obtained during extraction at different temperatures.

NMR data indicate that the ECPs and RCPs contains both proteinaceous and lignin matter. RCPs may also contain small amounts of carbohydrates. It has been proven that some DES has the unique capability to selectively extract lignin and hemicellulose from lignocellulosic biomass while cellulose being retained in the residue.37,38,43,57 The selective extraction of lignin alone or lignin and hemicellulose with DES described in the literature refers to biomass, such as wood,37,38 rice straw,58 wheat straw35 or corn stover,43,57 that contains little protein.

The presence of more phenolic compounds in ECPs may also be the cause of their dark color. Aromatic C signals in ECPs can come from lignin, but also from tannins (PA) and/or anthocyanin. Marles and Gruber47 reported that lignin concentration are important factors that may contribute to the concentration of PA in the seed coat, and lignin variability may influence pigment extractability. This is due to the position of the highly lignified palisade cells adjacent to the inner integument in the seed coat, where pigment is initially deposited.47 The good solubility of vegetable tannins in DES (choline chloride : ethylene glycol, 1:2 molar ratio) was described by Abbot *et al.* (2015).59 The presence of anthocyanin was found in seed60 and seedling61,62 in the *Oenothera* species, while they are absent in seed coat of the *Brassica* species.47 Isolation of anthocyanins with DES has been evidenced in the literature,26,63,64 also with Glyceline.26,64 However, to confirm that tannin and/or anthocyanins are responsible for coloring of evening primrose samples further studies should be carried out.

To the best of our knowledge, there are no papers on the use of Glyceline for the extraction of protein from plant materials. So far, the process for separating proteins and/or protein fractions from biomass materials using DES has been described in one patent,65 in which the claimants used DES comprising a hydrogen bond acceptor selected from alkali or earth alkaline metal carboxylates and urea as hydrogen bond donor.

**Elemental Analysis.** CHN analyses were performed for oil cakes, biomass residues and precipitates (Figure S3). Assuming that N content is solely due to proteinaceous matter, the protein content in the samples was calculated based on 6.25 conversion factor. As shown in Figures 5 and 6, the nitrogen content, and thus the protein content, is higher in precipitates than in the initial oil cakes. RCPs protein contents varied between 36-48%, where it increased with increasing temperature of the treatment. ECPs showed slightly higher values of protein, ranging from 40 to 50 %.

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**Figure 5**. Content of nitrogen (N) and protein of RC, RCBR and RCP.

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**Figure 6**. Content of nitrogen (N) and protein of EC, ECBR and ECP.

**SDS-Polyacylamide Gel Electrophoresis (SDS-PAGE).** SDS-PAGE of the oil cakes and precipitates are shown in Figure 7. SDS-PAGE of RC (Figure 7a) confirmed characteristic polypeptide bands originating from cruciferin and napin, similar to those described in the literature.66-68 Cruciferin gives several bands in the range of ~16-33 kDa, while napin gives two bands, ~11 kDa and ~7 kDa. SDS-PAGE of RCP60 and RCP100 shows characteristic bands for cruciferin, while for napin are absent. These data indicate that DES can selectively isolate the cruciferin from RC. This may be due to the different physico-chemical properties of cruciferin and napin. The napin shows more hydrophilic protein properties than cruciferin.10

In the case of evening primrose group, the characteristic polypeptide bands for EC are present in both ECP60 and ECP100 (Figure 7b). Results show that protein could be successfully extracted, however when biomass is treated at 140 °C, possible denaturation of the proteins occurs hence their separation by electrophoresis is hindered (Figure 7, RCP140 and ECP140).

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**Figure 7**. SDS-PAGE (under reducing conditions) pattern of RC, RCPs (a) and EC, ECPs (b) samples. MWM – molecular weight markers.

**Infrared Analysis (ATR-FTIR)**. The ATR-FTIR spectra of the feedstock (RC and EC), biomass residues after DES treatment (RCBRs and ECBRs), and precipitates (RCPs and ECPs) are shown in Figure 8, and the assignments are listed in Table S3.

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**Figure 8**. ATR-FTIR spectra of RC, RCBRs, RCPs (a) and EC, ECBRs, ECPs (b) samples.

The spectra of precipitates show strong absorption bands at 1625 cm-1 and 1534 cm-1 for RCPs and at 1648-1609 cm-1 and 1518 cm-1 for ECPs. These peaks can be assigned to amide I and amide II bands, respectively.70,71 It should be added that these bands can overlap with aromatic skeletal vibrations of lignin.72,73 In addition, amide I band can overlap with the band of absorbed water.74 These amide bands are more prominent in the precipitates than in the feedstocks.

The presence of lignin in ECPs can be confirmed by band at 1282 cm-1 and 1441 cm-1 which can be attributed to guaiacyl ring breathing with C−O stretching75,76 and aromatic ring vibrations,76 respectively. These bands are hardly visible in the RCP, ECBR and RCBR spectra. These results are consistent with the NMR data on which the signals from aromatic carbons are clearly visible on the ECP spectra, in contrast to the RCP, ECBR and RCBR.

The lignin absorption bands at the range 1200-1000 cm-1 are overlap with bands of carbohydrate and make specific assignment difficult. Nevertheless, there are differences in the spectra of precipitates and biomass residues in this region. A significant increase of bands in the region 1150-950 cm−1 is observed in ECBRs spectra, reflecting an increase of carbohydrates content in biomass after DES treatment. In the spectra of ECPs, bands in the range of 1000-950 cm−1 are negligible. This can infer that ECP does not contain significant amounts of carbohydrates, agreeing with the already discussed 13C CP-MAS NMR analysis. Absorption bands in the range of 1100-950 cm-1 in the spectra of RCPs indicates the presence of carbohydrates, which was also confirmed by NMR analysis. Given the poor solubility of cellulose in DES, it is most likely that these bands refer to hemicellulose and/or pectinaceous matter.

The absorption band at 1743 cm-1, corresponding to the carbonyl group, can be derived from hemicellulose, pectin and/or lignin. In ECPs and RCPs spectra the intensity of this band decrease in intensity as DES treatment temperature increases.

**Thermogravimetric Analysis (TGA).** The DTG curves of rapeseed and evening primrose sample groups are shown in Figures 9 and 10, respectively.

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**Figure 9**. DTG thermograms of RC and RCPs samples.

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**Figure 10**. DTG thermograms of EC and ECPs samples.

Starting with the rapeseed group (Figure 9), at least three major mass loss bands were identified. The first band, with a max. degradation rate (Td) around 80-100 °C, can be assigned to the loss of moisture and volatiles present in the samples, the second and third bands (Td ~340 °C and ~390 °C, respectively) may derive from the overlap of both, proteins and lignin decomposition patterns. However, it is known that rapeseed proteins have a major mass loss between 250-350 °C,77 which would correspond to the second major band, with Td ~340 °C. Also, the presence of a band with Td between 200-250 °C in the feedstock (RC, Figure 9) and in the biomass residues (Figures S6 and S7) suggests that polysaccharides (pectin, hemicellulose)49 present in RC are not extracted by DES, remaining in the final solid residue.

Similar pattern is observed for the evening primrose group (Figure 10), however, more lignin is presented in the ECPs than in RCPs, which is evidenced by the broad and slow-rate mass loss pattern with a Td around 400 °C, characteristic of lignin.78-81 The protein Td (around 350 °C) is overlapped by the lignin mass loss contribution.

**CONCLUSIONS**

As the global population is expected to increase to 9 billion by 2050, the need for global protein to feed a growing population will increase commensurately. New sources of protein will be needed beyond animal-derived. Agricultural residues and food supply chain wastes are expected to be interesting sources of vegetable protein to supplement global need.

The novel methodology used in this work demonstrates a suitable green alternative for the extraction of vegetable protein *via* pretreatment of oilseed cakes (rapeseed and evening primrose) with DES (Glyceline). Protein-rich precipitates were obtained by adding water (antisolvent) to the DES extract. The presence of proteins in precipitates has been confirmed by several techniques. The most promising results were obtained during the pretreatment of RC with DES. The precipitates obtained after treatment at 60 ˚C (RCP60) and 100 ˚C (RCP100) contain 4% and 9%, respectively, more protein compared to the initial rapeseed cake (RC). Cruciferin-rich RCP60 and RCP100 have the desired, light color, which can be much more favorably assessed by consumers than colored RC.

Proteins obtained from oil cakes (especially from rapeseed) can be an alternative to soy proteins, the most commonly used vegetable proteins. The use of the double-low variety of rapeseed (canola) can result in high quality food grade proteins. This work is undoubtedly a valuable source of information for producers of edible oils, biodiesel, pharmaceutical companies and other entities involved in the production of functional foods and nutraceuticals. Pretreatment of oilseed cake with natural products based-DES (Glyceline) open a new way to green extraction of vegetable protein with simultaneous possibility of reprocessed by-products in vegetable oil industry.

Deeper studies of precipitates are needed, including determination of undesirable compounds such as glucosinolates, phenolic compounds, dietary fiber and phytates to gain insight into the possibilities of their use for food applications. It is also very important to examine the functional properties (*e.g.* solubility, emulsifying ability, foaming properties, gel and film formation) of the precipitates obtained. This information can also be valuable due to the non-food application of vegetable proteins, *e.g*. for the production of films with water barrier properties, hydrogels, surfactants or bioplastics.6,82 In the future, the analysis of the composition of the solution separated from the precipitate, and the possibility of recovery and reuse of DES should be carried out. The challenge is also a sustainable management of biomass residues obtained after DES pretreatment.

**ASSOCIATED CONTENT**

**Supporting Information**

Instrumental experimental data, CHN, TG, DTG, 13C CP-MAS NMR and ATR-IR, chemical composition of rape and evening primrose seed and pressed cake based on literature data (PDF)

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**SYNOPSIS:** Green alternative for the extraction of vegetable protein *via* pretreatment of oilseed cakes (rapeseed and evening primrose) with DES (Glyceline)