International Journal of Technology 15(5) 1420-1437 (2024)
Received January 2024 / Revised February 2024 / Accepted May 2024

International Journal of Technology

http://ijtech.eng.ui.ac.id

Deep Eutectic Solvents and Natural Deep Eutectic Solvents for Extraction and Purification of Proteins from Animal and Botanical Sources - A Review

Andri Cahyo Kumoro[1,2*], Dyah Hesti Wardhani[1,2], Tutuk Djoko Kusworo[1], Mohammad Djaeni[1], Tan Chin Ping[3], Misbahudin Alhanif[1,2,4]

[1]*Department of Chemical Engineering, Faculty of Engineering, Universitas Diponegoro, Semarang, 50275, Indonesia*
[2]*Institute of Food and Remedies Biomaterials (INFARMA), Faculty of Engineering, Universitas Diponegoro, Semarang, 50275, Indonesia*
[3]*Department of Food Technology, Faculty of Food Science and Technology, Universiti Putra Malaysia, Serdang, 43400, Malaysia*
[4]*Chemical Engineering Study Program, Faculty of Industrial Technology, Institut Teknologi Sumatera, Lampung Selatan, 35365, Indonesia*

Abstract. The rising global population has led to an escalating demand for affordable, high-quality proteins intended for human consumption. Different efforts have been made to develop efficient and greener solvents for protein production. Most proteins are produced from raw plant and animal parts, but the use of the residues in commercial-scale protein production is very scarce. Therefore, this research aims to collect an overview of deep eutectic solvents (DESs) and natural deep eutectic solvents (NADESs) for protein extraction and purification. In this context, solvent type and operating parameters should also be selected to modify DESs and NADESs physicochemical characteristics for the achievement of high protein yield with preserved functional properties. The results show that appropriate implementation of combined DESs and NADESs with advanced extraction and purification techniques can improve protein yield and prevent detrimental effects on the extracted protein and environment. The application of DESs and NADESs can increase the efficiency of protein extraction and recovery in various parts of plants and animals by 55.72% and 98.16%, respectively, with purity reaching 99.82%. This research also reviews safety, environmental impacts, and drawbacks as well as shows feasible future recommendations for commercial-scale protein production processes. NADESs are safer than petroleum-based solvents but have higher toxicity than DESs. Magnetic adsorbent and magnetic solid-phase extraction methods have been shown to reduce labor-intensive steps, resulting in shorter operating times and superior protein recovery while maintaining functional properties. Meanwhile, protein producers' knowledge and motivation to use DES and NADES are strengthened.

Keywords: Animal and plant parts; Deep eutectic solvent; Extraction; Operating parameter; Protein

1. Introduction

Protein is the basic unit of living organisms acquired from sustainable origins, namely animals, plants, and microorganisms. Since the world's population is forecasted to reach 9 billion by 2050, the demand for affordable high-quality protein is expected to increase

*Corresponding author's email: andrewkomoro@che.undip.ac.id, Tel.: +62-24-7460058;
doi: 10.14716/ijtech.v15i5.6925

(Grudniewska *et al.*, 2018). Therefore, efficient, greener and sustainable methods for protein production receives a growing interest. The underused sources have also attracted attention for the preparation of affordable pure proteins. In this context, the food industries may also adopt underused plant and animal residues by extracting and refining proteins as valuable biomacromolecules showing numerous health benefits to reduce environmental problems and support sustainable development goals.

The crucial step in using protein is to adopt suitable extraction technologies to acquire protein from animal or plant matrices. Conventional procedures are techniques regularly used, such as chemical methods. However, the methods can lead to reduced extraction yields due to protein degradation (Kumar *et al.*, 2021b). Numerous industries also continue to depend on traditional methods because of financial viability. Chemical methods are categorized according to petroleum-based solvents, water, alkalis, organic solvents, and acids (Bowen *et al.*, 2022; Kumar *et al.*, 2021b). Meanwhile, chemical methods are used to enhance the retrieval of proteins (Kumar *et al.*, 2021a; Bose *et al.*, 2019). Protein purification methods include precipitation through the addition of an agent, electrophoresis, ion exchange, and affinity chromatography (Lin *et al.*, 2021). Conventional acid-based extraction is less promising due to lower quality of protein (Kumar *et al.*, 2021b). Petroleum-based and solvent-based chemical methods have been used in the food and pharmaceutical industries for centuries. However, this solvent has serious problems, those related to low extract yield and coextraction of unwanted substances, long extraction time, operational complexity, expensive, toxicity, biodegradability, flammability, safety and environmental sustainability, denaturation or reduced biological activities (Ling and Hadinoto, 2022; Fuad, Nadzir, and Harun, 2021; de Jesus and Filho, 2020).

Organic solvents are crucial in protein extraction and precipitation processes, leading to the production of pure products. Water-based extraction is a widely used method for obtaining proteins from different sources (Chen *et al.*, 2019a). The process is commonly adopted because of the elevated solubility and stability of isolated protein, which is mostly attributed to high content. Protein with the capacity of attaching to lipids have non-polar or polar side chains and contain aromatic amino acids that readily dissolve in organic solvents such as ethanol, butanol, and acetone (Qiaoyun *et al.*, 2017). Aqueous two-phase systems, created by combining polyethylene glycol (PEG) with salts can be used efficiently for purification and separation of proteins (Asenjo and Andrews, 2011).

Alkalis such as NaOH and KOH are frequently used to maintain a basic pH and enhance the yield compared to organic extraction. Altering the pH to a basic level caused the disulphide linkages to dissolve, leading to an enhancement in recovery and yield (Contreras *et al.*, 2019). The solubility is enhanced by an elevation in the pH of solvent due to the ionization of acidic and neutral amino acids at high pH levels. Therefore, extracting protein in an alkaline environment results in greater protein yields. In this context, a yield above 90% can be obtained from soybeans, rapeseed, and other seed commodities through alkaline extraction at high pH. Alkaline enhances protein extraction and disrupts the structure of amino acids, lysine and cysteine. This significantly degrades the digestibility and acceptability of the extracted protein (Kumar *et al.*, 2021b).

To address this limitation, the food industries have initiated the use of greener solvents as substitutes for petroleum-based solvents, alkalis, organic solvents, and acids to manufacture products (Ling and Hadinoto, 2022; Kumar *et al.*, 2021a; Kumoro *et al.*, 2019). In addition, ionic liquids (ILs), deep eutectic solvents (DESs) and analogues from natural sources (sugars, acids, and amino acids) known as natural deep eutectic solvents (NADESs) are progressing as advanced agents to decrease the adverse impacts of petroleum-based solvents (Ling and Hadinoto, 2022). DESs are composed of a quaternary ammonium salt

(QAS) and a hydrogen bond donor (HBD), such as a carboxylic acid or alcohol. These components are mixed in specific molar ratios to form a eutectic mixture with a lower melting point (Abbott *et al.*, 2003). Meanwhile, NADESs consist of natural compounds such as sugar (glucose), organic acids (citric acid), and amino acids (choline). This compound forms a eutectic mixture with properties similar to DES but derived from renewable resources (Dai *et al.*, 2013b).

Despite having comparable physical characteristics to ILs, such as high viscosity, low volatility, chemical and thermal stability, as well as non-flammability, DESs are not made of ionic compounds. Non-ionic chemicals can produce DESs (Ling and Hadinoto, 2022), which are more cost-effective with higher biodegradability (Kudłak, Owczarek, and Namieśnik, 2015). These solvents are environmentally friendly, easy to synthesize, cheap, biodegradable, and possess low toxicity (Chen *et al.*, 2018).

The use of DESs and NADESs as an environmentally friendly solvent for extracting valuable compounds has gained significant interest in recent years. This is due to the distinct characteristics, including customizable properties, versatility, ease of preparation, unique super-molecular structure that shows strong attraction to different compounds, high solubility, and stabilizing capability (Zannou and Koca, 2022; Dai *et al.*, 2013a). The majority of investigations on extraction have mostly focused on bioactive small compounds (Pratiwi *et al.*, 2020; de Faria *et al.*, 2017). Several reviews have been published on the latest developments in extraction of bioactive small compounds using DESs. Bonacci *et al.* (2020) gave an example showing the ability to extract DESs from tiny molecules such as phenolic compounds. The use of choline chloride glycerol DESs resulted in a significantly higher recovery yield of oleuropein (~88,287 ppm) from olive oil processing wastes. This was achieved in 10 minutes, which is twice the usual water extraction method requiring 30 minutes. The research reported by de Faria *et al.* (2017) also observed similar performance of DES. In this context, higher recovery of polyphenols and flavonoid compounds was obtained from saffron processing wastes compared to conventional solvents such as aqueous methanol, aqueous ethanol, and water. Mulia *et al.* (2015) have also succeeded in extracting α-mangostin with the highest yield of 2.6% (w/w) from dried mangosteen skin using NADESs. This was obtained using a mixture of choline chloride and 1,2-propanediol with a mole ratio of 1:3.

Even though DESs and NADESs extraction of small bioactive compounds is highly practical, the investigation of biological macromolecules, such as proteins, oils and carbohydrates, has gained attention (Ling and Hadinoto, 2022; Mulia *et al.*, 2018). Considering the distinct physicochemical characteristics of proteins in comparison to other small or large molecules, the function of DES and NADESs should be reported in extracting proteins derived from animals and plants. The selection of solvents depends on factors such as the solubility of protein, extraction efficiency, and desired properties of solvents. There are differences in protein from animals and plants due to variations in biochemical composition, cellular structure, and properties. Animal protein extraction includes methods such as aqueous, organic solvent, or enzymatic digestion, depending on the tissue type and desired protein (Malva *et al.*, 2018). Meanwhile, plant protein extraction requires additional steps to break down cell walls, such as mechanical disruption or enzymatic treatment, followed by aqueous or buffer solutions (Wang, Liu, and Lu, 2013). Extraction from animals and plants with greener and environmentally friendly solvents is interesting for further analysis.

In this research, extraction of DES and NADESs from animals and plants is presented. The review commenced with a discussion of the fundamental and physicochemical characteristics of DESs and NADESs suitable for protein extraction processes. The main

results and trends observed in solvent-based protein extraction were discussed with the safety and environmental aspects.

2. DESs and NADESs

Ionic liquids (ILs) are used as solvents for reaction or extraction processes and provide numerous advantageous characteristics, such as high boiling point, broad liquid range, selective dissolving capacity, excellent thermal stability, non-flammable and molecular structure variety (Morais *et al.*, 2020). However, the preparation is very complicated, requiring high equipment, manufacturing costs, and laborious recovery from the mixture with the target analyte (Ling and Hadinoto, 2022). To overcome the limitation, DESs are developed as a new generation of advanced greener solvents.

As a eutectic mixture, DES can be synthesized partially or completely from non-ionic compounds, which function as hydrogen bond donors (HBDs) such as betaine, choline chloride, guanidine hydrochloride and proline, as well as hydrogen bond acceptors (HBA) including amine groups (ethanolamine, dimethylamine, and imidazole), polyols (ethylene glycol, propylene glycol, and glycerol), and carboxylic acids (acetic, citric, maleic, and lactic acids) (Saini *et al.*, 2022). Meanwhile, NADESs consist of natural compounds such as sugar (glucose), organic acids (citric acid), or amino acids (choline). This compound forms a eutectic mixture with properties similar to DESs but derived from renewable resources (Dai *et al.*, 2013b). The preparation comprises sequential procedures, such as size reduction, heating, evaporation, and low-temperature drying (Saini *et al.*, 2022). After careful selection of the prerequisite substances, homogenization is carried out at ideal conditions (i.e., <100°C) to attain a eutectic mixture and equilibrate the resulting solution with ambient conditions (Mišan *et al.*, 2020). Therefore, the physicochemical characteristics of DESs are equivalent to ILs as a perfect substitute. The macromolecular structures are appropriate for protein extraction concerning solubility, affinity, and stability (Landa-Castro *et al.*, 2020). The influence of the composition of HBAs and HBDs on the characteristics is explained in Section 4.

DESs and NADESs can exist as binary or ternary mixtures. In addition, NADESs possess simpler preparation procedures, better efficiency, selectivity, biodegradability, thermal stability, and sustainability (Ijardar, Singh, and Gardas, 2022). Based on chemical compositions and formulas, DESs are grouped into four classes (Abbott *et al.*, 2004).

3. DESs and NADESs Physicochemical Characteristics Related to Extraction Process

Even though the physical characteristics of DESs are closely similar to the conventional ILs, the chemical characteristics are significantly different. The influential physicochemical characteristics are melting point, freezing temperature, polarity, solubility, miscibility, pH, density, viscosity, interfacial tension, refraction index, and ionic conductivity (Omar and Sadeghi, 2022b). The anion size, equilibrium molar ratio of HBA/HBD, alkyl chain length, and molecular mass of HBA/HBD at the melting point determine the physicochemical characteristics (Omar and Sadeghi, 2022a). However, viscosity and polarity possess significant effects on extraction performance (Tolmachev *et al.*, 2022). Interfacial tension also has a remarkable effect on liquid-liquid and ultrasound-assisted extraction processes (Kumoro *et al.*, 2022).

The fluidity of DESs is mainly characterized by viscosity (Omar and Sadeghi, 2022b), influencing the solubility of target samples. In this context, the biomacromolecules are more soluble in less-viscous DESs. Temperature, type of HBAs and HBDs, HBA/HBD molar ratio, and molecular mass greatly affect viscosity. Meanwhile, an increase in temperature

appreciably decreases viscosity due to hydrogen bond network cleavage between HBA and HBD as well as the reduction of internal resistance of molecules (Ling and Hadinoto, 2022). DESs possess higher viscosity when sugar, carboxylic acid and metallic compounds are selected as the HBD, while less viscous type can be prepared using ethylene glycol, glycerol, and phenol (Ling and Hadinoto, 2022). After water addition, the viscosity decreases, facilitating better mixing and mass transfer leading to a higher extraction performance. An extreme amount of water ceases the advantageous characteristics of DES. Therefore, an appropriate water quantity should be selected to prevent the disruption of hydrogen bond interactions among DES-forming substances (Ling *et al.*, 2020).

Polarity, which is closely related to Hansen's solubility parameter (HSP) is a theoretical method used as an introductory tool to estimate the solubility of target biomacromolecules in different solvents and select the most appropriate DESs and NADESs for extraction from respective natural sources (El-Kantar *et al.*, 2019). Since DESs are mostly polar substances, the superior ability to dissolve the target analytes can improve extraction yield (Gullón *et al.*, 2020). Eutectic solvent polarity is attributed to the HBDs used for the synthesis and the highest are usually synthesized from carboxylic acids. In contrast, polyols and sugars result in eutectic solvents with the lowest polarity (Xu *et al.*, 2019a). The dilution of DESs with water beyond 50% causes more hydrogen bond destruction and leads to a decrease in extraction ability (Dai *et al.*, 2015).

The density of DESs is higher than pure water (Omar and Sadeghi, 2022b), with the value ranging from 1.0 to 1.35 g/cm^3 at 25^{o}C. However, the density of metallic salts containing DESs lies between 1.3 to 1.6 g/cm^3 (Zhang *et al.*, 2012), depending on the molecular arrangement, temperature, HBA/HBD molar ratio, as well as the presence of cavities and voids. Temperature greatly decreases the density, refraction index, and acoustic velocity due to the enhancement of ionic movement and unoccupied volume (Omar and Sadeghi, 2022b).

Surface tension is an important characteristic of DESs utilization in the interface and colloid system (Kumoro *et al.*, 2022). In this context, HBDs and HBAs have an appreciable influence on the surface tension of DESs. Since DESs are highly hydrophobic, the surface tension is enhanced greatly when the water mole fraction is higher than 0.9 (Chen *et al.*, 2019b). However, the surface tension decreases as a function of solvent concentration when petroleum-based solvents or crystal water in the salt component are added with an increasing temperature from 20 to 60°C (Chen *et al.*, 2019b).

The majority of DESs are highly viscous liquids with large ion sizes and possess low ionic conductivity (below 1 mS/cm at ambient temperature) which limits the application in isoelectric-based protein precipitation (Omar and Sadeghi, 2022b). The ionic conductivity is influenced by alkyl chain length of cation, the characteristics of organic salt, HBDs and HBAs ionic ratio, temperature, and water content (Dai *et al.*, 2015). Therefore, the conductivity can be improved by increasing the free volume through the reduction of cation size and the substitution of HBD with fluorinated substances to reduce viscosity (Abbott, Capper, and Gray, 2006). Another strategy is to increase the temperature which helps to break the hydrogen bond network and increase ionic motility (Omar and Sadeghi, 2022b). The tunable physical characteristics, specifically density, viscosity, ionic conductivity, and freezing point obtained by a careful selection of biodegradable HBD and HBA couplings make DESs and NADESs more attractive for specific applications (Zhang *et al.*, 2012).

4. DESs and NADESs as Extracting Media for Protein Extraction and Purification

DESs, formed by the combination of hydrogen bond donors and acceptors, have been extensively studied for the versatile applications. For instance, Abbott *et al.* (2003) showed

the exceptional solvent properties of DESs, particularly in extraction of bioactive compounds from natural sources. The results showed that DESs, such as those composed of choline chloride and urea, reported superior extraction yields and selectivity compared to conventional solvents. Similarly, Dai *et al.* (2013a) suggested the efficiency in catalyzing various chemical reactions, showing the potential as green reaction media. Conversely, NADESs are derived from naturally occurring compounds such as sugars, organic acids, and amino acids, and the efficiency has been reported in various processes. For example, Socas-Rodríguez *et al.* (2021) investigated the use as extraction solvents for bioactive compounds from different matrices. The research showed that NADESs offered a greener alternative to conventional solvents, with comparable or improved extraction efficiency. Generally, DESs and NADESs offer promising prospects as efficient and greener solvents across various industries. The unique properties, coupled with the renewable and sustainable nature, position solvents as viable alternatives, contributing to the development of more sustainable processes.

Efficiency and yield when using DES or NADES can vary depending on the specific application and the characteristics of solvent system. These eco-friendly solvents offer several advantages contributing to improved efficiency and yield in various processes, including extraction, synthesis, and catalysis. According to Smith, Abbott, and Ryder (2014), solvents possess unique properties in enhancing the efficiency and yield of chemical processes. The properties include the ability to enhance solubility, provide selective extraction, operate under mild reaction conditions, and offer recyclability. By leveraging the characteristics, DES and NADES have shown potential to facilitate higher yields and improved efficiency compared to conventional solvents (Smith, Abbott, and Ryder, 2014).

To elaborate on a specific example, extraction of bioactive compounds is considered from plant material using NADESs. Choi *et al.* (2011) reported the use in understanding cellular metabolism and physiology, emphasizing the potential for efficient extraction of target compounds from natural sources. The selected method includes preparing the NADESs by mixing components such as choline chloride and organic acids in specific ratios, followed by extraction of bioactive compounds from plant material at an appropriate temperature. The resulting extract can be analyzed using analytical techniques to quantify yield and purity. Moreover, Dai *et al.* (2015) discussed the tailoring of properties, including the addition of water, to optimize performance in various applications. This optimization process enhances efficiency and yield by fine-tuning solvent characteristics to suit specific extraction or synthesis requirements.

In the case of protein extractions, the most used DESs belong to type III and comprise ChCl as HBA and amines, amide, carboxylic acids, sugars, and polyols as HBD (Zhang *et al.*, 2012). Ling and Hadinoto (2022) showed that there were considerable variations in melting points when the HBA:HBD ratios in ChCl:urea were changed. A 1:2 ratio resulted in a significantly lower melting temperature of 12^{o}C, while a 1:1 ratio generated NADESs with a high melting point of 50^{o}C. Therefore, the choice of HBD component has a major impact on the melting point of the resulting DES as well as the effect of the molar ratio of HBA and HBD. For example, the use of ethylene glycol, citric acid, malonic acid, oxalic acid, xylitol, and glycerol as HBD produced DESs with melting temperatures ranging from 69 °C to room temperature. In this context, the type of HBD affects the melting point of the synthesized DESs (Zhang *et al.*, 2012). An appropriate HBA/HBD molar ratio in the mixture plays a crucial role in ensuring the suitability of the resulting DESs to selectively dissolve the targeted proteins and easy solvent recovery.

4.1. DESs and NADESs for Solid–Liquid Extraction of Protein from Plant and Animal Parts

The mechanism of DESs and NADESs in solid-liquid extraction of proteins from plant and animal parts includes a series of steps. These solvents penetrate the cell structures of plant materials or animal tissues, disrupting hydrogen bonds and hydrophobic interactions (Morais *et al.*, 2020). Within plant or animal material, DESs and NADESs solubilize proteins through interactions such as hydrogen bonding and electrostatic interactions (Bowen *et al.*, 2022). Meanwhile, extraction methods such as shaking or stirring enhance protein leaching from the solid matrix into solvent phase (Zhou, Fakayode and Li, 2023). Subsequent phase separation techniques, such as centrifugation or filtration, isolate protein-containing solvent phase from the solid debris (García *et al.*, 2015). Protein recovery from solvent phase is also achieved through precipitation or purification methods, with the possibility of solvent regeneration for further use (Abbott *et al.*, 2004). This mechanism shows the potential of DESs and NADESs as sustainable alternatives for protein extraction, offering applications in various industries including food, pharmaceuticals, and biotechnology.

Different investigations have also focused on the usage of biomass residues as tabulated in Table 1. These greener solvents have proven the ability to achieve higher extraction performance in terms of product yield and purity. Liu *et al.* (2017) prepared DESs by mixing several HBAs, such as ChCl, glycine, betaine, alanine chloride, acetylcholine chloride, and nicotinic acid with PEG200 to extract pumpkin seeds protein. The PEG200-based DESs were mixed with a four-fold volume of ethanol as well as 1 M hydrochloric acid to control the pH at 4.5 and recover 93.8% of the extracted protein (Table 1). Currently, extraction of proteins using PEG-based DESs is growing more rapidly. This is because the extracted proteins are more stable and accepted by Food and Drug Administration (FDA), which opens wider opportunities for more sustainable developments in the agricultural, food and pharmaceutical sectors (Morgenstern *et al.*, 2017). Even though PEG-based DESs show excellent affinity to protein as well as a remarkable capacity to ease precipitation, more research is required to ensure the scale-up and practical application at a commercial scale.

Hernández-Corroto *et al.* (2020) used highly polar and hydrophilic DESs derived from ChCl and acetic acid to extract pomegranate peel protein. The resulting extract contained 19.2 mg protein/g with high antihypertensive activity, which was stronger than petroleum-based extract. After selecting nine ChCl – diol mixtures as DESs, Yue *et al.* (2021) found that ChCl–1,4-butanediol/water mixture was the perfect solvent for oat protein extraction with an efficiency of 55.72% as well as better stability and foaming capacity (Table 1). DESs derived from multicomponent mixtures could precipitate protein more rapidly than binary mixtures caused by high partitioning capacity and polarity.

Chen *et al.* (2021) prepared DESs by mixing ChCl with glycerol to extract soy protein with a yield of 10% higher than the acid-based precipitation method, which reported enhanced heat resistance and hydrophobicity. An equivalent observation was stated by Lin *et al.* (2021) when acidic DESs based on ChCl and levulinic acid were used to extract protein from bamboo shoots. The research observed a more profound protein yield enhancement (60%) compared to extraction using sodium hydroxide solution.

With the intention of marine by-product valorization, Rodrigues *et al.* (2021) prepared DES by blending betaine and propylene glycol in a ratio of 1:3 to recover proteins from sardine fish heads and entrails. A yield of 162.2 mg protein/g fish parts greater than the common aqueous extraction was obtained. This product contained numerous hydrophobic amino acids, namely alanine, isoleucine, leucine, and valine that are applicable to produce less polar DESs. In addition, the resulting extracts showed stronger antioxidant and antimicrobial capacities. The existence of hydrophobic DESs components promotes more

intensive interactions between the respective proteins with the cell membrane of the assayed microorganisms.

Rodrigues *et al.* (2021) extracted protein from sardine processing residue using betaine–propylene glycol and obtained a yield of 162.2 mg protein/g fish with higher antioxidant and antimicrobial activities than the water extract. DESs made from lactic acid and L-cysteine are capable of recovering keratin from coarse wool residue without altering the polypeptide structure (Okoro *et al.*, 2022). Based on those reviewed facts, DESs and NADESs are capable of extracting valuable proteins and preserving desirable functional characteristics.

Table 1 Extraction of proteins from sustainable sources using various DESs and NADESs

NADESs/DESs	Protein Sources	Process Parameters	Yields	References
Solid-liquid extraction (SLE)				
ChCl–based NADES with ethylene glycol	orange peel	Temp.: 4°C, time: 15 min	7.7 mg/g FW	(Panić *et al.*, 2021)
ChCl–levulinic acid	basal bamboo shoot (BBS), sheath, tip bamboo shoot (TBS)	Temp.: 80°C, time: 50 min, solid/liquid ratio (30 mg/mL)	Protein: 15.46 ± 0.30 mg/g DW, 9.54 ± 0.17 mg/g DW and 39.16 ± 1.22 mg/g DW for BBS, sheath and TBS.	(Lin *et al.*, 2021)
ChCl–butanediol	oat	Temp.: 80°C, time: 1.5 h	Total protein yield: 55.72%, extracted protein has high solubility, foaming capacity, and stability.	(Yue *et al.*, 2021)
ChCl–glycerol	soybean	Temp.:60°C, time: 3.9 h, liquid/solid ratio: 10.3, stirring speed: 873 rpm, water content: <15 wt.%	Total protein yield: 34.62%, extracted protein has good heat resistance and is highly hydrophobic.	(Chen *et al.*, 2021)
ChCl–acetic acid	pomegranate peels	Time: 15 min, ChCl:AA molar ratio 1:2	Protein yield: 19.2 mg/g of protein with excellent antihypertensive capacity	(Hernández-Corroto *et al.*, 2020)
ChCl–PEG	pumpkin seed	Temp.: 43°C, liquid/solid ratio: 28 mL/g, microwave power: 140 W, DES concentration: 28% w/w, precipitation time: 4 min	Protein yield: 93.95% (w/w) of protein	(Liu *et al.*, 2017)
Betaine–propylene glycol (B:PG)	Sardine processing residues	Temp.:80°C, time: 18 h, B:PG molar ratio: 1:3, liquid/solid ratio: 80	Total protein yield: 162.2 mg/g protein.	(Rodrigues *et al.*, 2021)
Liquid-liquid extraction (LLE)				
ChCl–glycerol	BSA	Temp.: 30°C, DES: 1.3 g, salt solution concentration: 0.9 g/mL	98.16% of BSA was transferred into the DES-rich phase of ATPS, back-extraction efficiency:32.96%	(Xu *et al.*, 2016)
Tetrabutylammonium bromide–glycolic acid	Lysozyme from chicken egg white	Temp.: 35°C, DES <1.0 g, salt solution concentration < 0.25 g/mL	98.16% of lysozyme was transferred into the DES-rich phase of ATPS, 91.73% of the initial activity of lysozyme was retained	(Xu *et al.*, 2019b)
[TBAC][PPG400]/ [Pro][Xyl]	Chymotrypsin	Temp.: 35°C, [Pro][Xyl]: 1.6 g, Protein: 8 mg, [TBAC][PPG400]: 1.0 g	extraction efficiency: 97.30%	(Meng *et al.*, 2019)

There are many parameters, which provide essential roles during biomacromolecules extraction, specifically proteins using DESs and NADESs, such as temperature, duration, solvent-to-solid ratio, solid particle size, and pH (Kumoro *et al.*, 2022). An appropriate selection of temperature, water content, and pH will alter polarity, solubility, interfacial tension, and viscosity supporting the achievement of high extraction performance (Huang *et al.*, 2017).

4.2. DESs for Liquid-Liquid Extraction of Protein from Plant and Animal Parts

Aqueous two-phase systems (ATPS) are used to facilitate selective liquid-liquid extraction of proteins by homogenizing a water-soluble polymer with another inorganic salt, such as PEG-salt-water mixture and ethylene oxide–propylene oxide or copolymer–polyoxyethylene detergent at a concentration higher than the critical value (Xu *et al.*, 2016). Xu *et al.* (2016) prepared an ATPS by mixing ChCl and glycerol with a salt solution and successfully extracted 98.16% of bovine serum albumin (BSA) from the DESs phase without any protein conformation changes. Hydrogen bonding, salting out, and hydrophobic interactions are possible characteristics facilitating protein uptake (Xu *et al.*, 2016).

Xu *et al.* (2019b) recovered lysozyme (Lyz) from chicken egg white using DESs-based ATPS derived from tetrabutylammonium bromide (TBAB), glycolic acid (Gly) and Na_2SO_4 salt. More than 98% of the lysozyme was transferred to the DESs-phase with 91.73% of the initial activity preserved. Similarly, Meng *et al.* (2019) selected a mixture of tetrabutylammonium chloride (TBAC), L-proline – xylitol (Pro– Xyl) of 1:6 and polypropylene glycol 400 as DESs and ATPS to extract chymotrypsin from the mixture with BSA and lysozyme. The result also showed that 97.30% chymotrypsin was accumulated in the [Pro][Xyl]-rich phase under optimum conditions (pH 7.0, 35^{o}C, 12 minutes shaking). The phase separation capacity of DESs can be improved by enhancing the alkyl side chain length of carboxylic acids and the addition of a benzyl group.

Liquid-liquid extraction of proteins using DESs and NADESs in combination with ATPS includes a sophisticated mechanism. Solvents penetrate the cellular structures of plant materials or animal tissues, disrupting intermolecular forces and solubilizing proteins (Abbott *et al.*, 2003). Subsequently, the mixture is combined with an aqueous solution to form a biphasic system, where protein partitions between the two phases based on the physicochemical properties (Singh and Tavana, 2018). Liquid-liquid extraction methods such as stirring or shaking facilitate the transfer of protein into solvent-rich phase, while the remaining sample matrix remains in the aqueous phase. Subsequent separation methods, such as centrifugation or inversion isolate protein-enriched phase from the aqueous phase (Mendes *et al.*, 2023). Protein recovery can be achieved through precipitation or purification methods, with the potential for solvent regeneration for reuse. This integrated method obtains the advantages of DESs and NADESs in combination with ATPS to offer an efficient and sustainable method for protein extraction from diverse biological sources.

4.3. Protein Purification Using DESs and NADESs

The extracted proteins must be purified to allow food, pharmaceutical, and nutraceutical industries to commercialize safe products for actual human and animal consumption. The well-established methods to purify protein are alkali, ammonium sulphate or acetone precipitation, salting-out, ion exchange, electrophoresis, and affinity chromatography (Sindhu *et al.*, 2012). However, the established techniques suffer from limitations, such as protein activity loss, denaturation or complexation, higher operating costs, and equipment operation difficulty. For example, an enzyme's activity can be distorted by excessive interaction with a polar solvent. In this context, a smart purification

strategy using ATPS can be a potential alternative method due to shorter purification and phase separation time, excellent capacity to preserve biological activity, great biocompatibility, low toxicity, and lower requirement of water (Gai *et al.*, 2011). DESs have also been used as a part of ATPS to purify protein (Table 2) (Zeng *et al.*, 2016).

Purification process using ATPS offers a versatile method for separating biomolecules based on differential partitioning between two immiscible aqueous phases (Albertsson, 1970). This strategy includes the design of phase-forming components such as polyethylene glycol (PEG) and dextran to optimize protein partitioning behavior (Singh and Tavana, 2018). After mixing the sample with ATPS, the target protein preferentially partitions into a phase while impurities remain in the other, facilitating efficient separation (Hatti-Kaul, 2000). Smart technologies integrated into purification process enable real-time monitoring of protein, allowing for precise adjustment of conditions to enhance separation efficiency (Bernau *et al.*, 2022). Meanwhile, separation techniques such as centrifugation are used to isolate the phases containing the purified protein, followed by further purification steps when necessary (Du *et al.*, 2022). By obtaining automation and data-driven optimization, this ATPS-based strategy ensures high yields and purity levels in biotechnology and pharmaceutical applications.

Table 2 Utilization of DES-based ATPS for protein purification

	ATPS	Proteins	Purification rate (%)	References
Associated with DES	Betaine: glycerol: H_2O (1: 2: 1) -K_2HPO_4	BSA	99.82	(Li *et al.*, 2016)
	ChCl: glycerol (1: 1)-K_2HPO_4	BSA	98.71	(Xu *et al.*, 2016)
	ChCl: glycerol (1: 1)-K_2HPO_4	Trypsin	94.36	(Xu, Wang, and Hou, 2020)
	ChCl: urea (1: 2)-K_2HPO_4	R-phycoerythrin	92.60	Xu, Wang, and Hou, 2020)
Protein purification by MSPE	PEG4000–$MgSO_4$	BSA	82.68	(Saravanan *et al.*, 2008)
	Betaine–K_2HPO_4	BSA	90	(Zeng *et al.*, 2016)

To achieve the objective, Li *et al.* (2016) adopted betaine as the HBA to compose six types of DESs using urea, methyl urea, glucose, sorbitol, glycerol, and ethylene glycol as HBD to extract and purify BSA from protein mixture. In this context, betaine – urea mixture was found as the most favorable DESs in combination with ATPS for BSA extraction and purification from the complex systems where an efficiency of 99.82% was achieved. Moreover, Xu *et al.* (2015) used DESs derived from ChCl as HBA and ethylene glycol, glycerol, glucose, and sorbitol as HBD to extract and purify BSA from the mixture. The result confirmed that ChCl–glycerol mixture at a 1:1 molar ratio was the most favorable DESs. Under optimum pH, temperature, and time, BSA and trypsin recoveries were 98.71% and 94.36%, respectively. Meanwhile, Xu, Wang, and Hou (2020) observed that ChCl–urea (1:2) mixture was the most preferred DESs to extract R-phycoerythrin from red algae. A ternary mixture of ChCl–urea–K_2HPO_4 was the suitable ATPS to purify R-phycoerythrin with a separation efficiency of 92.60% (Xu, Wang, and Hou, 2020).

5. Limitations of DESs and NADESs as Proteins Extraction and Purification Media

Even though many chemicals comply with the functions of HBD and HBA to form DESs, some of solvents are not appropriate for protein extraction from natural sources. Therefore, a careful selection should be carried out to determine the appropriate DESs, specifically

those related to recovery and isolation from the phase (Smith, Abbott, and Ryder, 2014). DESs viscosities and densities decline with the increase in temperature but the ionic conductivity rises (Lores *et al.*, 2017). These opposite features can be the critical problems of viability and adaptability potentially affecting the utilizations by the industry.

Efficient recovery and isolation are essential for protein separation from DESs to enable recycling. Xu *et al.* (2015) observed that recovery from this solvent was slow since the process occurred under a mass transfer regime due to high interfacial resistance. Even though salt concentration is modified by mixing DESs with a freshly ethanolic saline solution mixture, only 32.9% of protein is recovered. Therefore, more advanced improvements should be addressed to the existing protein back extraction and DESs recovery techniques to attract more interest from enterprises (Li *et al.*, 2016).

6. Safety and Environmental Aspects Associated with The Utilization of DESs and NADESs

NADESs are less toxic than petroleum-based solvents, and the adverse effects on human health remain unclear but solvents are regarded as safe (GRAS). A previous in vivo research on mice and Wistar rats confirmed that NADESs reported higher toxicity than DESs, which was rooted in high viscosity values. At high concentrations, solvent becomes very viscous limiting the circulation and inducing acute effects, liver failure, and mortality (Benlebna *et al.*, 2018). Ecotoxicity research of DESs reported disparate sensitivities to the tested ecosystem models, which mainly varied with the composition of DESs. The results showed that DESs and NADESs toxicity was affected by some parameters, namely viscosity, HBD/HBA molar ratio, organic acids content, pH, type of cells or organisms, and synergistic effects.

Previous safety analysis is required before using DESs and NADESs for food and medicinal applications. Since the characteristics largely depend on the combinations of the components, a compendious database of toxicological properties of DESs should be set up which supports the numerous component variations of the mixtures with computational predictive approximations. In addition, most NADESs combinations and applications are patented limiting the industrial applications.

7. New Methods for Solid-Phase Extraction Using DESs and NADESs

The direct utilization of DESs and NADESs using traditional extraction methods can collaborate with ultrasound-assisted (UAE) and microwave-assisted extraction (MAE) to achieve higher protein yield and purity. The application can be expected to extensively break cell wall structure and ease the release of intracellular protein from plant and animal matrices (Chemat *et al.*, 2019). Correspondingly, DESs can also be developed in MAE for the recovery of proteins and show greater performances than conventional techniques (Bubalo *et al.*, 2016). This pilot strategy possesses various advantages mainly related to higher efficiency, shorter operation time, and lower solvent requirement than conventional extraction methods using petroleum-based solvents.

To enhance extraction performance, magnetic adsorbents can be incorporated into ATPS, which function to adsorb proteins for pure protein recovery (Liu *et al.*, 2012). In magnetic solid-phase extraction (MSPE), nanoparticles are dispersed into extraction medium to adsorb proteins. The analytes are immediately segregated from the magnetic adsorbents with the aid of an external magnetic field enabling nearly complete recovery of protein molecules and recycling of nano adsorbing particles (Table 3) (Wen *et al.*, 2016). This innovative method eliminates some lengthy and laborious stages, namely

centrifugation and filtration, that result in a shorter operating time, and exceptional protein recovery with preserved functional properties and offers an important function in purification procedures (Huang *et al.*, 2015).

Table 3 Application of magnetic particle-modified DESs for protein purification

Target Protein	Magnetic particle	DESs/ NADESs	Extraction capacity ($mg.g^{-1}$)	References
chymotrypsin	Fe_3O_4@TiO_2	[ChCl][Xyl](1: 1)	347.8	(Li *et al.*, 2021)
R-phycoerythrin	MB-NH_2@CD	[BeCh][Tri](1: 2)	549.87	(Xu, Wang, and Hou, 2020)
BSA	Fe_3O_4-NH_2@GO	[ChCl] [glycerol] (1: 1)	44.59	(Xu *et al.*, 2015)
BSA	M-CNT@	N-[APTMAC][Xyl](1: 1)	225.15	(Ni *et al.*, 2020)
BSA	Fe@GO @Amino functional dicationic ionic liquid		89.7	(Wen *et al.*, 2016)
BSA	Fe@GO		6.7	

8. Conclusions

In conclusion, DESs and NADESs were reported to show higher extraction efficiency, improved bioactivity, better recyclability, reusability and biodegradability potential, as well as less toxicity than conventional organic solvents. The collaboration of solvents with green chemistry-based industrial processes to reclaim valuable substances from plant and animal sources reported more favourable results compared to the available conventional solvent extraction processes. In addition, some important process parameters, namely solid and solvent ratio, temperature, mixing, pH, and duration, significantly affected the effectiveness of DESs during extraction processes. From a scale-up and industrial application perspective, the use of DESs required further research concerning thermal stability, analyte purification, solvent recovery, operational cost, toxicity, and environmental impacts.

Acknowledgments

The authors acknowledge Universitas Diponegoro for its financial assistance under the third-year term of World Class Research Universitas Diponegoro (Kategori A) with contract No.: 118-17/UN7.6.1/PP/2021.

References

Abbott, A.P., Boothby, D., Capper, G., Davies, D.L., Rasheed, R.K., 2004. Deep Eutectic Solvents Formed between Choline Chloride and Carboxylic Acids: Versatile Alternatives to Ionic Liquids. *Journal of the American Chemical Society,* Volume 126(29), pp. 9142–9147

Abbott, A.P., Capper, G., Davies, D.L., Rasheed, R.K., Tambyrajah, V., 2003. Novel Solvent Properties of Choline Chloride/Urea Mixtures. *Chemical Communications,* Volume 1, pp. 70–71

Abbott, A.P., Capper, G., Gray, S., 2006. Design of Improved Deep Eutectic Solvents Using Hole Theory. *Chemphyschem: a European Journal of Chemical Physics and Physical Chemistry,* Volume 7(4), pp. 803–806

Albertsson, P.-Å., 1970. Partition of Cell Particles and Macromolecules in Polymer Two-Phase Systems. *Advances in Protein Chemistry,* Volume 24, pp. 309–341

Asenjo, J.A., Andrews, B.A., 2011. Aqueous Two-Phase Systems for Protein Separation: A Perspective. *Journal of Chromatography A,* Volume 1218(49), pp. 8826–8835

Benlebna, M., Ruesgas-Ramón, M., Bonafos, B., Fouret, G., Casas, F., Coudray, C., Durand, E., Cruz Figueroa-Espinoza, M., Feillet-Coudray, C., 2018. Toxicity of Natural Deep Eutectic Solvent Betaine:Glycerol in Rats. *Journal of Agricultural and Food Chemistry,* Volume 66(24), pp. 6205–6212

Bernau, C.R., Knödler, M., Emonts, J., Jäpel, R.C., Buyel, J.F., 2022. The Use of Predictive Models to Develop Chromatography-Based Purification Processes. *Frontiers in Bioengineering and Biotechnology,* Volume 10, pp. 1–24

Bonacci, S., Di Gioia, M.L., Costanzo, P., Maiuolo, L., Tallarico, S., Nardi, M., 2020. Natural Deep Eutectic Solvent as Extraction Media for The Main Phenolic Compounds from Olive Oil Processing Wastes. Antioxidants, Volume 9(6), p. 513

Bose, U., Broadbent, J.A., Byrne, K., Hasan, S., Howitt, C.A., Colgrave, M.L., 2019. Optimisation of Protein Extraction for In-Depth Profiling of The Cereal Grain Proteome. *Journal of Proteomics,* Volume 197, pp. 23–33

Bowen, H., Durrani, R., Delavault, A., Durand, E., Chenyu, J., Yiyang, L., Lili, S., Jian, S., Weiwei, H., Fei, G., 2022. Application of Deep Eutectic Solvents in Protein Extraction and Purification. *Frontiers in Chemistry,* Volume 10, pp. 1–10

Bubalo, M.C., Ćurko, N., Tomašević, M., Kovačević-Ganić, K., Radojčić-Redovniković, I., 2016. Green Extraction of Grape Skin Phenolics by Using Deep Eutectic Solvents. *Food Chemistry,* Volume 200, pp. 159–166

Chemat, F., Abert-Vian, M., Ravi, H.K., Khadhraoui, B., Hilali, S., Perino, S., Tixier, A.-S.F., 2019. Review of Alternative Solvents for Green Extraction of Food and Natural Products: Panorama, Principles, Applications and Prospects. *Molecules (Basel, Switzerland),* Volume 24(16), p. 3007

Chen, J., Jiang, X., Yang, G., Bi, Y., Liu, W., 2018. Green and Efficient Extraction of Resveratrol from Peanut Roots Using Deep Eutectic Solvents. *Journal of Chemistry,* Volume 2018(1), p. 4091930

Chen, Q., Chaihu, L., Yao, X., Cao, X., Bi, W., Lin, J., Chen, D.D.Y., 2021. Molecular Property-Tailored Soy Protein Extraction Process Using a Deep Eutectic Solvent. *ACS Sustainable Chemistry & Engineering,* Volume 9(30), pp. 10083–10092

Chen, R., Wang, X.-J., Zhang, Y.-Y., Xing, Y., Yang, L., Ni, H., Li, H.-H., 2019a. Simultaneous Extraction and Separation of Oil, Proteins, And Glucosinolates from Moringa Oleifera Seeds. *Food Chemistry,* Volume 300, p. 125162

Chen, Y., Chen, W., Fu, L., Yang, Y., Wang, Y., Hu, X., Wang, F., Mu, T., 2019b. Surface Tension of 50 Deep Eutectic Solvents: Effect of Hydrogen-Bonding Donors, Hydrogen-Bonding Acceptors, Other Solvents, and Temperature. *Industrial & Engineering Chemistry Research,* Volume 58(28), pp. 12741–12750

Choi, Y. H., van Spronsen, J., Dai, Y., Verberne, M., Hollmann, F., Arends, I.W.C.E., Witkamp, G.J., Verpoorte, R., 2011. Are Natural Deep Eutectic Solvents the Missing Link in Understanding Cellular Metabolism and Physiology? *Plant Physiology,* Volume 156(4), pp. 1701–1705

Contreras, M. del M., Lama-Muñoz, A., Manuel Gutiérrez-Pérez, J., Espínola, F., Moya, M., Castro, E., 2019. Protein Extraction from Agri-Food Residues for Integration in Biorefinery: Potential Techniques and Current Status*. Bioresource Technology,* Volume 280, pp. 459–477

Dai, Y., van Spronsen, J., Witkamp, G.-J., Verpoorte, R., Choi, Y.H., 2013a. Natural Deep Eutectic Solvents as New Potential Media for Green Technology. *Analytica Chimica Acta,* Volume 766, pp. 61–68

Dai, Y., Witkamp, G.-J., Verpoorte, R., Choi, Y.H., 2013b. Natural Deep Eutectic Solvents as a New Extraction Media for Phenolic Metabolites in Carthamus Tinctorius L. *Analytical Chemistry,* Volume 85(13), pp. 6272–6278

Dai, Y., Witkamp, G.-J., Verpoorte, R., Choi, Y.H., 2015. Tailoring Properties of Natural Deep Eutectic Solvents with Water to Facilitate Their Applications. *Food Chemistry,* Volume 187, pp. 14–19

de Faria, E.L.P., do Carmo, R.S., Cláudio, A.F.M., Freire, C.S.R., Freire, M.G., Silvestre, A.J.D., 2017. Deep Eutectic Solvents as Efficient Media for the Extraction and Recovery of Cynaropicrin from Cynara Cardunculus L. Leaves. *International Journal of Molecular Sciences,* Volume 18(11), p. 2276

De Jesus, S.S., Filho, R.M., 2020. Recent Advances in Lipid Extraction Using Green Solvents. *Renewable and Sustainable Energy Reviews,* Volume 133, p. 110289

Du, M., Hou, Z., Liu, L., Xuan, Y., Chen, X., Fan, L., Li, Z., Xu, B., 2022. Progress, Applications, Challenges and Prospects Of Protein Purification Technology. *Frontiers in Bioengineering and Biotechnology,* Volume 10, pp. 1–26

El-Kantar, S., Rajha, H. N., Boussetta, N., Vorobiev, E., Maroun, R. G., Louka, N., 2019. Green Extraction of Polyphenols from Grapefruit Peels Using High Voltage Electrical Discharges, Deep Eutectic Solvents and Aqueous Glycerol. *Food Chemistry,* Volume 295, pp. 165–171

Fuad, F.M., Nadzir, M.M., Harun, A., 2021. Hydrophilic Natural Deep Eutectic Solvent: A Review on Physicochemical Properties and Extractability of Bioactive Compounds. *Journal of Molecular Liquids,* Volume 339, p. 116923

Gai, Q., Qu, F., Zhang, T., Zhang, Y., 2011. Integration of Carboxyl Modified Magnetic Particles and Aqueous Two-Phase Extraction for Selective Separation of Proteins. *Talanta,* Volume 85(1), pp. 304–309

García, G., Aparicio, S., Ullah, R., Atilhan, M., 2015. Deep Eutectic Solvents: Physicochemical Properties and Gas Separation Applications. *Energy & Fuels,* Volume 29(4), pp. 2616–2644

Grudniewska, A., de Melo, E.M., Chan, A., Gniłka, R., Boratyński, F., Matharu, A.S., 2018. Enhanced Protein Extraction from Oilseed Cakes Using Glycerol–Choline Chloride Deep Eutectic Solvents: A Biorefinery Approach. *ACS Sustainable Chemistry & Engineering,* Volume 6(11), pp. 15791–15800

Gullón, P., Gullón, B., Romaní, A., Rocchetti, G., Lorenzo, J.M., 2020. Smart Advanced Solvents for Bioactive Compounds Recovery from Agri-Food By-Products: A Review. *Trends in Food Science & Technology,* Volume 101, pp. 182–197

Hatti-Kaul, R., 2000. Methods and Protocols: Methods and Protocols. Humana Press, pp. 1–10

Hernández-Corroto, E., Plaza, M., Marina, M.L., García, M.C., 2020. Sustainable Extraction of Proteins and Bioactive Substances from Pomegranate Peel (Punica Granatum L.) Using Pressurized Liquids and Deep Eutectic Solvents. *Innovative Food Science & Emerging Technologies,* Volume 60, p. 102314

Huang, Y., Feng, F., Jiang, J., Qiao, Y., Wu, T., Voglmeir, J., Chen, Z.-G., 2017. Green and Efficient Extraction of Rutin from Tartary Buckwheat Hull by Using Natural Deep Eutectic Solvents. *Food Chemistry,* Volume 221, pp. 1400–1405

Huang, Y., Wang, Y., Pan, Q., Wang, Y., Ding, X., Xu, K., Li, N., Wen, Q., 2015. Magnetic Graphene Oxide Modified with Choline Chloride-Based Deep Eutectic Solvent for The Solid-Phase Extraction of Protein. *Analytica Chimica Acta,* Volume 877, pp. 90–99

Ijardar, S.P., Singh, V., Gardas, R.L., 2022. Revisiting the Physicochemical Properties and Applications of Deep Eutectic Solvents. *Molecules*, Volume 27(4), p. 1368

Kudłak, B., Owczarek, K., Namieśnik, J., 2015. Selected Issues Related to The Toxicity of Ionic Liquids and Deep Eutectic Solvents--A Review. *Environmental Science and Pollution Research International*, Volume 22(16), pp. 11975–11992

Kumar, M., Potkule, J., Patil, S., Saxena, S., Patil, P.G., Mageshwaran, V., Punia, S., Varghese, E., Mahapatra, A., Ashtaputre, N., Souza, C.D., Kennedy, J.F., 2021a. Extraction of Ultra-Low Gossypol Protein from Cottonseed: Characterization Based on Antioxidant Activity, Structural Morphology and Functional Group Analysis. *LWT,* Volume 140, p. 110692

Kumar, M., Tomar, M., Potkule, J., Verma, R., Punia, S., Mahapatra, A., Belwal, T., Dahuja, A., Joshi, S., Berwal, M.K., Satankar, V., Bhoite, A.G., Amarowicz, R., Kaur, C., Kennedy, J.F., 2021b. Advances in The Plant Protein Extraction: Mechanism and Recommendations. *Food Hydrocolloids*, Volume 115, p. 106595

Kumoro, A.C., Hasan, M., Singh, H., 2019. Extraction of Andrographolide from Andrographis paniculata Dried Leaves Using Supercritical CO2 and Ethanol Mixture. *Industrial & Engineering Chemistry Research,* Volume 58(2), pp. 742–751

Kumoro, A.C., Wardhani, D.H., Kusworo, T.D., Djaeni, M., Ping, T.C., Azis, Y.M.R.F., 2022. Fish Protein Concentrate for Human Consumption: A Review of Its Preparation By Solvent Extraction Methods And Potential For Food Applications. *Annals of Agricultural Sciences,* Volume 67(1), pp. 42–59

Landa-Castro, M., Sebastián, P., Giannotti, M.I., Serrà, A., Gómez, E., 2020. Electrodeposition of Nanostructured Cobalt Films From a Deep Eutectic Solvent: Influence Of The Substrate And Deposition Potential Range. *Electrochimica Acta,* Volume 359, p. 136928

Li, H., Wang, Y., He, X., Chen, J., Xu, F., Liu, Z., Zhou, Y., 2021. A Green Deep Eutectic Solvent Modified Magnetic Titanium Dioxide Nanoparticles for The Solid-Phase Extraction of Chymotrypsin. *Talanta,* Volume 230, p. 122341

Li, N., Wang, Y., Xu, K., Huang, Y., Wen, Q., Ding, X., 2016. Development of Green Betaine-Based Deep Eutectic Solvent Aqueous Two-Phase System for The Extraction of Protein. *Talanta,* Volume 152, pp. 23–32

Lin, Z., Jiao, G., Zhang, J., Celli, G.B., Brooks, M.S.-L., 2021. Optimization of Protein Extraction From Bamboo Shoots and Processing Wastes Using Deep Eutectic Solvents in A Biorefinery Approach. *Biomass Conversion and Biorefinery,* Volume 11(6), pp. 2763–2774

Ling, J.K., Hadinoto, K., 2022. Deep Eutectic Solvent as Green Solvent in Extraction of Biological Macromolecules: A Review. *International Journal of Molecular Sciences,* Volume 23(6). p. 3381

Ling, J.K.U., Chan, Y.S., Nandong, J., Chin, S.F., Ho, B.K., 2020. Formulation of Choline Chloride/Ascorbic Acid Natural Deep Eutectic Solvent: Characterization, Solubilization Capacity and Antioxidant Property. *LWT,* Volume 133, p. 110096

Liu, Q., Shi, J., Cheng, M., Li, G., Cao, D., Jiang, G., 2012. Preparation of Graphene-Encapsulated Magnetic Microspheres for Protein/Peptide Enrichment and MALDI-TOF MS analysis. *Chemical Communications,* Volume 48(13), pp. 1874–1876

Liu, R.-L., Yu, P., Ge, X.-L., Bai, X.-F., Li, X.-Q., Fu, Q., 2017. Establishment of an Aqueous PEG 200-Based Deep Eutectic Solvent Extraction and Enrichment Method for Pumpkin (Cucurbita moschata) Seed Protein. *Food Analytical Methods,* Volume 10(6), pp. 1669–1680

Lores, H., Romero, V., Costas, I., Bendicho, C., Lavilla, I., 2017. Natural Deep Eutectic Solvents in Combination with Ultrasonic Energy As A Green Approach for Solubilisation of Proteins: Application To Gluten Determination By Immunoassay. *Talanta,* Volume 162, pp. 453–459

Malva, A. Della, Albenzio, M., Santillo, A., Russo, D., Figliola, L., Caroprese, M., Marino, R., Díaz-Cruz, J.M., 2018. Methods for Extraction of Muscle Proteins from Meat and Fish Using Denaturing and Nondenaturing Solutions. *Journal of Food Quality,* Volume 2018, p. 8478471

Mendes, M.S.M., Rosa, M.E., Ramalho, F., Freire, M.G., e Silva, F.A., 2023. Aqueous Two-Phase Systems as Multipurpose Tools to Improve Biomarker Analysis. *Separation and Purification Technology,* Volume 317, p. 123875

Meng, J., Wang, Y., Zhou, Y., Chen, J., Wei, X., Ni, R., Liu, Z., Xu, F., 2019. Development of Different Deep Eutectic Solvent Aqueous Biphasic Systems for The Separation Of Proteins. *RSC Advances*, Volume 9(25), pp. 14116–14125

Mišan, A., Nađpal, J., Stupar, A., Pojić, M., Mandić, A., Verpoorte, R., Choi, Y.H., 2020. The Perspectives of Natural Deep Eutectic Solvents in Agri-Food Sector*. Critical Reviews in Food Science and Nutrition,* Volume 60(15), pp. 2564–2592

Morais, E.S., Lopes, A.M.D.C., Freire, M.G., Freire, C.S., Coutinho, J.A., Silvestre, A.J., 2020. Use of Ionic Liquids and Deep Eutectic Solvents In Polysaccharides Dissolution And Extraction Processes Towards Sustainable Biomass Valorization. *Molecules,* Volume 25(16), p. 3652

Morgenstern, J., Baumann, P., Brunner, C., Hubbuch, J., 2017. Effect of PEG Molecular Weight and PEGylation Degree on the Physical Stability of PEGylated Lysozyme. *International Journal of Pharmaceutics,* Volume 519(1–2), pp. 408–417

Mulia, K., Adam, D., Zahrina, I., Krisanti, E.A., 2018. Green Extraction of Palmitic Acid from Palm Oil using Betaine-based Natural Deep Eutectic Solvents. *International Journal of Technology*. Volume 9(2), pp. 335–-344

Mulia, K., Krisanti, E., Terahadi, F., Putri, S., 2015. Selected Natural Deep Eutectic Solvents for the Extraction of α-mangostin from Mangosteen (Garcinia mangostana L.) Pericarp. *International Journal of Technology,* Volume 7(1), pp. 22–30

Ni, R., Wang, Y., Wei, X., Chen, J., Meng, J., Xu, F., Liu, Z., Zhou, Y., 2020. Magnetic Carbon Nanotube Modified with Polymeric Deep Eutectic Solvent for The Solid Phase Extraction of Bovine Serum Albumin. *Talanta,* Volume 206, p. 120215

Okoro, O. V., Jafari, H., Hobbi, P., Nie, L., Alimoradi, H., Shavandi, A., 2022. Enhanced keratin Extraction from Wool Waste Using A Deep Eutectic Solvent. *Chemical Papers*, Volume 76(5), pp. 2637–2648

Omar, K.A., Sadeghi, R., 2022a. Novel Lacmoid-Based Deep Eutectic Solvent Dye as Writing Ink. *Chemical Engineering Research and Design,* Volume 180, pp. 50–54

Omar, K.A., Sadeghi, R., 2022b. Physicochemical Properties of Deep Eutectic Solvents: A Review. *Journal of Molecular Liquids,* Volume 360, p. 119524

Panić, M., Andlar, M., Tišma, M., Rezić, T., Šibalić, D., Cvjetko Bubalo, M., Radojčić Redovniković, I., 2021. Natural Deep Eutectic Solvent as A Unique Solvent for Valorisation of Orange Peel Waste by The Integrated Biorefinery Approach. *Waste Management,* Volume 120, pp. 340–350

Pratiwi, F.A., Utami, T.S., Arbianti, R., 2020. Using Ultrasonic Assisted Extraction to Produce a Bioinsecticide from Cigarette Butt Waste and Green Solvent to Control Armyworm Infestation. *International Journal of Technology,* Volume 11(7), pp. 1329–1336

Qiaoyun, C., Xinghong, N., Liang, Z., Zheng, T., Jin, L., Kang, S., Xuan, C., Xinghui, L., 2017. Optimization of Protein Extraction and Decoloration Conditions for Tea Residues. *Horticultural Plant Journal*, Volume 3(4), pp. 172–176

Rodrigues, L.A., Leonardo, I.C., Gaspar, F.B., Roseiro, L.C., Duarte, A.R.C., Matias, A.A., Paiva, A., 2021. Unveiling the Potential Of Betaine/Polyol-Based Deep Eutectic Systems For

The Recovery of Bioactive Protein Derivative-Rich Extracts From Sardine Processing Residues. *Separation and Purification Technology,* Volume 276, p. 119267

Saini, A., Kumar, A., Panesar, P.S., Thakur, A., 2022. Potential of Deep Eutectic Solvents in The Extraction of Value ‐ Added Compounds from Agro ‐ Industrial By ‐ Products. *Applied Food Research*, Volume 2(2), p. 100211

Saravanan, S., Rao, J.R., Nair, B.U., Ramasami, T., 2008. Aqueous Two-Phase Poly(Ethylene Glycol)–Poly(Acrylic Acid) System For Protein Partitioning: Influence of Molecular Weight, pH and Temperature. *Process Biochemistry*, Volume 43(9), pp. 905–911

Sindhu, R., Binod, P., Janu, K.U., Sukumaran, R.K., Pandey, A., 2012. Organosolvent Pretreatment and Enzymatic Hydrolysis of Rice Straw for The Production of Bioethanol. *World Journal of Microbiology & Biotechnology*, Volume 28(2), pp. 473–483

Singh, S., Tavana, H., 2018. Collagen Partition in Polymeric Aqueous Two-Phase Systems for Tissue Engineering. *Frontiers in Chemistry,* Volume 6, pp. 4–10

Smith, E.L., Abbott, A.P., Ryder, K.S., 2014. Deep Eutectic Solvents (DESs) and Their *Applications. Chemical Reviews,* Volume 114(21), pp. 11060–11082

Socas-Rodríguez, B., Torres-Cornejo, M.V., Álvarez-Rivera, G., Mendiola, J.A., 2021. Deep Eutectic Solvents for The Extraction of Bioactive Compounds from Natural Sources and Agricultural By-Products. *Applied Sciences*, Volume 11(11), p. 4897

Tolmachev, D., Lukasheva, N., Ramazanov, R., Nazarychev, V., Borzdun, N., Volgin, I., Andreeva, M., Glova, A., Melnikova, S., Dobrovskiy, A., Silber, S. A., Larin, S., de Souza, R. M., Ribeiro, M. C., Lyulin, S., Karttunen, M., 2022. Computer Simulations of Deep Eutectic Solvents: Challenges, Solutions, and Perspectives. *International Journal of Molecular Sciences,* Volume 23(2), p. 645

Wang, W., Liu, X., Lu, X., 2013. Engineering Cyanobacteria to Improve Photosynthetic Production of Alka(E)Nes. *Biotechnology for Biofuels*, Volume 6(1), p. 69

Wen, Q., Wang, Y., Xu, K., Li, N., Zhang, H., Yang, Q., Zhou, Y., 2016. Magnetic Solid-Phase Extraction of Protein by Ionic Liquid-Coated Fe@Graphene Oxide. *Talanta,* Volume 160, pp. 481–488

Xu, K., Wang, Y., Huang, Y., Li, N., Wen, Q., 2015. A Green Deep Eutectic Solvent-Based Aqueous Two-Phase System for Protein Extracting. *Analytica Chimica Acta*, Volume 864, pp. 9–20

Xu, M., Ran, L., Chen, N., Fan, X., Ren, D., Yi, L., 2019a. Polarity-Dependent Extraction of Flavonoids from Citrus Peel Waste Using A Tailor-Made Deep Eutectic Solvent. *Food Chemistry,* Volume 297, p. 124970

Xu, P., Wang, Y., Chen, J., Wei, X., Xu, W., Ni, R., Meng, J., Zhou, Y., 2019b. Development of Deep Eutectic Solvent-Based Aqueous Biphasic System for The Extraction of Lysozyme. *Talanta,* Volume 202, pp. 1–10

Xu, P., Zheng, G.-W., Du, P.-X., Zong, M.-H., Lou, W.-Y., 2016. Whole-Cell Biocatalytic Processes with Ionic Liquids. *ACS Sustainable Chemistry & Engineering*, Volume 4(2), pp. 371–386

Xu, Y., Wang, Q., Hou, Y., 2020. Efficient Purification of R-phycoerythrin from Marine Algae (Porphyra yezoensis) Based on a Deep Eutectic Solvents Aqueous Two-Phase System. *Marine Drugs,* Volume 18(12), p. 618

Yue, J., Zhu, Z., Yi, J., Lan, Y., Chen, B., Rao, J., 2021. Structure and Functionality of Oat Protein Extracted by Choline Chloride–Dihydric Alcohol Deep Eutectic Solvent and Its Water Binary Mixtures. *Food Hydrocolloids,* Volume 112, p. 106330

Zannou, O., Koca, I., 2022. Greener Extraction of Anthocyanins and Antioxidant Activity from Blackberry (Rubus spp) Using Natural Deep Eutectic Solvents. LWT, Volume 158, p. 113184

Zeng, C.-X., Xin, R.-P., Qi, S.-J., Yang, B., Wang, Y.-H., 2016. Aqueous Two-Phase System Based on Natural Quaternary Ammonium Compounds for The Extraction of Proteins. *Journal of Separation Science,* Volume 39(4), pp. 648–654

Zhang, Q., De Oliveira Vigier, K., Royer, S., Jérôme, F., 2012. Deep Eutectic Solvents: Syntheses, Properties and Applications. *Chemical Society Reviews*, Volume 41(21), pp. 7108–7146

Zhou, M., Fakayode, O.A., Li, H., 2023. Green Extraction of Polyphenols via Deep Eutectic Solvents and Assisted Technologies from Agri-Food By-Products. *Molecules,* Volume 28(19), p. 6852