Sustainable Extraction Technology Using Deep Eutectic Solvents for Producing Fava Bean Protein Isolates with Improved Functionalities

By

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Abstract

The fava bean is a legume crop belonging to the *Fabaceae* family. It has a long history of being used as food for human consumption. The fava bean is cultivated in various locations, but in countries with more advanced economies like Canada, it is generally considered an underutilized food for human consumption; thus, production primarily focuses on animal feed. Nutritionally, fava beans are a rich protein source, making them an ideal choice for producing protein ingredients. The limitations of conventional plant protein extraction methods have driven increasing interest in novel, sustainable green extraction technologies. Therefore, the main objectives of this research were to (1) develop a novel protein extraction method using Deep Eutectic Solvents (DES) and physiochemical characterization of extracted fava bean protein isolates (FBPI), (2) evaluate the structure-functional properties and protein quality of DES-FBPI, (3) employ the integrated pre-treatment-assisted DES extraction methods to enhance the structure-functional properties of FBPI, (4) determine the effect of different water substitution in DES on physiochemical characterization and structure-functional properties of DES-FBPI, and (5) understand the fava bean protein's gelation mechanism influenced by the DES extraction. As the first objective, the DES system was optimized for the extraction variables using the response surface methodology (RSM). The optimum conditions achieved indicated that the DES could produce a significantly higher protein yield (~65%) with similar protein content (~92%) to the conventional alkaline method (AE). The secondary structure components analysis revealed that DES extraction was less prone to protein denaturation than AE. Owing to these properties of DES-FBPI, the functional properties, solubility, and emulsifying and foaming properties were greatly improved. Interestingly, the DES method produced high-quality protein with a higher proportion of essential amino acids (EAA), including more sulfur-containing amino acids. This was reflected

in the significantly higher protein digestibility-corrected amino acid score (PDCAAS), which was greater than 75% compared to the protein obtained from AE and salt extraction. Using ultrasonication and enzymatic pre-treatment as an integrated approach, the efficiency of DES extraction substantially increased the protein yield, with improved functionality, including solubility, water-holding capacity, and fat-holding capacity. However, no significant effect on the foaming and emulsifying properties was observed. Despite DES's widely recognized sustainable characteristics, viscosity is a major drawback in industrial applications. The different water substitution levels applied in the DES system (0-100% wt%) resulted in significantly reduced viscosity, thereby significantly increasing protein yield. The DES-water system, containing 40 wt% hydration levels, preserved the supramolecular structure while gradually weakening the hydrogen bond network. Beyond the 40 wt% water addition, complete dissociation of DES components was revealed. These physicochemical and structural changes led to significant differences in the structure-functional properties of the extracted proteins. The behavior of fava bean protein in the DES system is crucial in determining protein functionality, such as the gelation mechanism. In particular, the gelling capacity of DES-FBPI was a distinguishable characteristic, producing a strong, firm gel at a pH of 7.0 with a protein concentration of 20% w/v, compared to commercial soy protein isolates (CS-PI). Predominantly, the DES-FBPI gels were stabilized by hydrophobic interactions and disulfide bonds and, to a lesser extent, by hydrogen and electrostatic interactions. Hence, it is noteworthy that DES extraction offers a valuable opportunity to replace soy-based foods with fava bean proteins as a potential strategy to reduce soy allergenicity. In conclusion, DES extraction presents a viable alternative to conventional methods, offering green benefits, enhanced protein quality, and improved techno-functional properties. Its scalability at the

industrial level could increase the utilization of fava beans in value-added food formulations, while these findings lay the groundwork for further research.

Dedication

In dedication to my ever-loving parents and family for their unconditional love and support

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Thesis format

This thesis has been prepared in manuscript format. The reference style follows the 7th edition of the American Psychological Association (APA) guidelines. The thesis has eight chapters, of which Chapter 1 provides the introduction, and Chapter 2 is a published literature review. Chapters 3, 4, 5, 6, and 7 are experimental chapters, with Chapter 3 containing the published manuscript. The remainder of each chapter consists of manuscripts prepared for publication. Chapter 8 summarizes the main findings of the research, provides a general discussion, and concludes with recommendations for future research.

Experimental chapters and contributions of authors

Chapter 2: Novel Extraction technologies for developing plant protein ingredients with improved functionality

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Chapter 3: Improved protein extraction technology using deep eutectic solvent system for producing high-purity fava bean protein isolates at mild conditions

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- Dr. Oladipupo Odunayo Olatunde- Reviewing and editing
- Dr. Chamila Nimalaratne: Reviewing and editing
- Dr. James D. House: Amino acid data generation, reviewing, and editing
- Dr. Rotimi Aluko: Reviewing and editing
- Dr. Nandika Bandara: Conceptualization, supervision, reviewing, editing, and provision of funding.

Chapter 4: Effects of novel protein extraction methods on structure-functional properties and protein quality of fava bean protein isolates: A comparative study.

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- Anuruddika Hewage: Designing the experiment, data analysis, writing the original draft, reviewing, and editing
- Dr. Amanda G.A. Sa – Amino acid and protein digestibility data generation, reviewing, and editing
- Dr. Rotimi Aluko: Reviewing and editing

- Dr. James D. House: Amino acid data and protein digestibility generation, reviewing, and editing
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Chapter 6: Effect of water addition on structure and functionality of fava bean proteins extracted by choline chloride and glycerol-based deep eutectic solvent system

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- Dr. Michel Aliani: NMR data generation, reviewing and editing
- Dr. Rotimi Aluko: Reviewing and editing
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Chapter 7: Understanding the fava bean protein gelation and its mechanism: Effect of novel deep eutectic solvents (DES) and conventional extraction methods on protein gelation.

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- Anuruddika Hewage: Designing the experiment, data analysis, writing the original draft, reviewing, and editing
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- Dr. Deepak Kadam: SEC data generation, reviewing and editing
- Dr. Filiz Koksel: Rheological data generation, reviewing and editing
- Dr. Rotimi. E. Aluko: Reviewing and editing
- Dr. Nandika Bandara: Conceptualization, supervision, reviewing, editing, and provision of funding.

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Chapter 1

General Introduction, Hypothesis, and Objectives

1.1. Introduction

The demand for plant proteins among consumers has increased tremendously over recent years. This is expected to increase further due to shifting dietary patterns toward plant-based diets. For example, the global protein ingredients market was valued at 61.0 billion in 2023 and is expected to continue in the coming years (Global Protein Ingredients Market Size Report, 2021-2028). As a result, the need for alternative proteins to satisfy consumers' nutritional requirements has become crucial in the current food sector. Although soy protein products currently dominate the Canadian plant-based proteins market, Canada is one of the world's leading producers of legume crops such as peas, chickpeas, lentils, and beans (Bolek & Gray, 2019). Nevertheless, most products are exported without further processing for value addition. With Canada's abundant agricultural resources and infrastructure, there is significant potential to expand domestic value-added operations using these emerging protein-rich legume crops.

The fava bean is one of the most globally important valuable legume crops in the Fabaceae family, grown in diverse environments (Sharan et al., 2021). Nutritionally, the fava bean is a rich source of protein, ranging from 25% to 40% (Augustin & Cole, 2022; Valente et al., 2018). The top fava bean producers in the global context are China, Ethiopia, Egypt, Sudan, the United Kingdom, Australia, Germany, France, and Morocco (Khazaei et al., 2019). In 2021, nearly 3.05 million ha were utilized globally (in 65 countries) for producing dry and green fava bean seeds (FAO statistics, 2023). In North America, fava bean production increased sporadically in the early 1970s but has not expanded to the same extent as lentils and peas (Vandenberg & Khazaei, 2023). However, since then, fava beans have been largely targeted for livestock feeding, with smaller quantities for human consumption. (Vandenberg & Khazaei, 2023). This is mainly due to the content of antinutritional factors like vicine and convicine, and the large seed sizes. On the other

hand, it is challenging for Canadian fava beans to compete with other exporters due to their lower quality. For example, Egypt requires medium to large seed sizes with high tannin content for human consumption and fractionated protein ingredients. Domestically, fava beans are now being processed into fractionation, albeit in relatively small quantities at a slower market pace (Global Economic Outlook for Faba Beans & Soybeans, Saskatchewan Pulse Growers, 2024).

Industrial protein fractionation is generally accomplished through the solubilization of the flour at a higher pH (alkaline medium), followed by isoelectric acid precipitation (Gençdağ et al., 2021). The fava bean has been well studied for protein extraction and functions using the conventional methods in the literature (Krause et al., 2023; Vogelsang-O'Dwyer et al., 2020) and the results were promising as an alternative plant protein to fill the protein demand. Nevertheless, AE accompanies several concerns related to environmental pollution via wastewater production, high water consumption, and protein denaturation, which subsequently affect protein functions (Hewage et al., 2022; Sun & Bandara, 2019). Under these circumstances, the food industry seeks sustainable techniques to produce high-quality proteins. This has become more challenging for the industry as consumer perception and demand for sustainable, healthy options for their dietary regimen have increased.

Currently, some advanced technologies, such as ultrasound, microwaves, high hydrostatic pressure, enzymatic treatment, and pulse electric field, have been successfully used for extraction with high protein yields and short extraction times (Jain et al., 2024; Rashwan et al., 2025). However, the commercialization of these techniques is still in its infancy, and the cost associated with implementing these novel techniques would be very high (Pojić et al., 2018). Therefore, an eco-friendly, sustainable, green solvent method alternative to organic solvents would be more beneficial.

Deep eutectic solvents (DES) are a novel class of green solvents, first discovered by Abbott and his co-workers (Abbott et al., 2003) in 2003. They synthesized the DES by mixing choline chloride and urea at a molar ratio of 1:2, whose melting point was 12 °C, which is lower than that of the urea (~302 °C) and the choline chloride (~133 °C). Such reduction was believed to be charge delocalization through hydrogen bonding between urea molecules and the chloride ion (Abbott et al., 2004). The resulting eutectic mixture was found to possess superior solvent properties compared to those of ionic liquids. Generally, DESs are synthesized by mixing two or more components: a hydrogen bond donor (HBD) and a hydrogen bond acceptor (HBA). Upon heating and mixing with an appropriate molar ratio, it forms an eutectic mixture characterized by an extensive network of hydrogen bonds whose melting point is lower than either individual component (Chen & Mu, 2019; Hansen et al., 2021). DES systems can be prepared from a binary mixture of HBD, including amide, amino acid, sugars, alcohol, amine, polyols, or carboxylic acid, and an HBA, such as a quaternary ammonium salt (Abbott et al., 2004; Florindo et al., 2018). DESs are widely recognized due to their sustainable characteristics: lower ecological footprint, low toxicity, recyclability, low cost, high tunability, easy preparation, no purification requirement, low volatility, and high chemical and thermal stability make it an incipient sustainable solvent with many increasing applications in electrochemistry, organic synthesis, biocatalysis, biodiesel preparation, metal-catalyzed or metal mediated organic reactions, and extractions (Chen & Mu, 2019; Hansen et al., 2021; Hewage et al., 2022).

Initially, the application of DES in protein extraction was limited, but considerable research on the extraction of bioactive molecules using DES has been reported (Airouyuwa et al., 2025; Dardavila et al., 2023; Ozel et al., 2024). Later, it expanded into protein extraction, owing to its green chemistry principles. So far, limited studies have been conducted on protein extraction using

DESs, among which different DES systems were reported for oats (Yue et al., 2021), canola (Karimi et al., 2024), bamboo shoots (Lin et al., 2020), soybean (Chen et al., 2021), tiger nut meal (Jiang et al., 2024), seabuckthorn meal (Lin et al., 2022), evening primrose, and rapeseed meal (Grudniewska et al., 2018). Generally, the findings of these studies remarked that comparatively lower protein yield (< 50%) and protein content (60%-70%) when compared with the proteins obtained from the AE method. Furthermore, studies related to techno-functional properties have not been well investigated. The fundamental aspects of protein function are prerequisites for determining food processing and product quality. Therefore, further research is essential to address the major gaps in DES protein extraction, employing a systematic approach to understand DES's fundamental behavior in relation to protein structure.

One of the limitations arising from novel protein extractions from plants is the interactions between proteins and other cellular components, including lipids and polysaccharides, which diminish the extraction efficiency (Rahman & Lamsal, 2021). Plant proteins are found in various forms and locations within the cell, and extraction becomes more challenging due to the plant's rigid cell wall (Görgüç et al., 2019); therefore, cell destruction is necessary to liberate bound and storage proteins. Recent developments in novel technologies can be successfully integrated into green extraction methods as a novel approach to enhance extraction efficiency. Among these technologies, enzymatic and ultrasonication (US) techniques stand out as promising methods due to their environmental friendliness, non-toxic nature, reduced energy consumption, minimal chemical waste generation, and ease of application in a short time (Rahman & Lamsal, 2021; Tang et al., 2024). Previously reported studies revealed that enzymes facilitate the breakdown of cellular structures and enhance protein solubility, leading to higher extraction yields and improved functional properties of the isolated proteins (Koysuren et al., 2021; Perović & Antov, 2022). The

US extraction is primarily dependent on cavitation, in which bubbles are formed and then gradually increase in volume and finally explode, releasing large amounts of mechanical and thermal energy, which benefits cell wall damage, facilitating efficient solvent penetration through improved cell permeability (Eze et al., 2022). This enhances the liberation of cell constituents into the extraction media, increasing the protein yield. Recently, research studies have demonstrated that US and enzymatic-assisted pretreatments resulted in higher extraction yields and superior functionality of proteins than the conventional methods (Gulzar et al., 2024; Orellana-Palacios et al., 2022; Wang et al., 2020). Therefore, the synergistic effect of green technologies is crucial for exploring innovative extraction methods for plant-based proteins across various food processing streams. Due to the limited capacity of reported data on combining green technologies with DES extraction, further research is needed to provide novel insights for the industry's future food applications.

Despite the sustainable solvent properties of DES, some barriers hinder industrial adaptation. The unique, extensive hydrogen bond network between the DES components renders the eutectic mixture highly viscous, which impedes both mass transfer rates and protein solubility, resulting in poor extraction efficiency (Chunyan Ma et al., 2018). Additionally, the reduced flow of high-viscosity DES requires high energy to pump throughout the vessel system at large-scale production, and cleaning would incur an extra cost for clogged pumps due to the highly viscous material. Therefore, adding water is vital to the DES to reduce viscosity and circumvent these bottlenecks. This would increase the mass transfer rate of protein molecules and reduce the high chemical consumption. However, adding water changes the physiochemical properties of DES, including viscosity, density, conductivity, and surface tension, thereby altering protein molecular dynamics (Dong et al., 2023). Hence, water substitution can modulate the DES physiochemical

properties tailored to the specific industrial application. Therefore, exploring specific designer DES systems for a specific protein function is useful in novel food formulations.

The technical and scientific aspects of food processing play a key role in determining the novel food formulations. The innovative protein extraction technology, such as DES, has great potential in bringing novel food formulations and could be linked to the production of sustainable protein ingredients, for example, food gelation. Investigating the gelling properties and behavior of DES-extracted proteins would provide new directions for replacing soy protein-based products, which likely involve developing restructured foods with adequate mechanical integrity, nutritional value, and desirable consumer acceptability. Hence, fundamental aspects of the gelling behavior of DES-extracted proteins should be studied in depth to understand the molecular interactions and gel strength, thereby enabling better utilization in future food formulations.

Therefore, we envisage that novel protein ingredient production using DES extraction from fava beans provides valuable insights for academia to expand the exploration of plant protein extraction and adapt it for industry, thereby enhancing fava bean utilization in novel food formulations.

Hence, the following hypotheses and research objectives are laid down to investigate fundamental aspects of DES extraction from fava bean proteins.

1.2. Hypothesis

1) DES extraction would improve the protein yield, protein content, and recovery rate of fava bean compared to the conventional alkaline extraction method.
2) DES extraction would affect the physiochemical properties of fava bean proteins differently, lowering the denaturation degree compared to conventional extraction methods.

3) DES extraction would enhance the techno-functional properties and fava bean protein quality in terms of amino acid composition and *in-vitro* protein digestibility.
4) Ultrasound and enzymatic pre-treatment assisted DES extraction would further increase the protein yield, protein content, and the techno-functional properties of fava bean proteins
5) Different percentages of water substitution in the DES system would affect its physiochemical and structural properties, including protein yield, recovery, and techno-functional properties, which would positively affect tailoring the DES system for specific food applications.
6) Compared to conventional extraction techniques, DES-extracted fava bean protein isolates would exhibit improved gelling properties, including gel strength and gel structure.

1.3. Objectives

The primary objective of this research study is to use novel sustainable DES extraction technology to produce fava bean protein ingredients and evaluate their techno-functional properties for food applications. The following sub-objectives are garnered to

a) Optimize the DES protein extraction variables using the response surface statistical model.
b) Determine the protein yield and recovery rate of DES-extracted fava bean protein isolates (DES-FBPI) compared to the conventional alkaline extraction method.
c) Characterize and evaluate techno-functional properties of DES-FBPI in comparison to conventional alkaline and salt extraction methods and commercial soybean protein as a standard.
d) Employ the pre-treatment-assisted DES extraction to improve the protein yield and techno-functional properties in DES-FBPPI.
e) Investigate the effect of water substitution in DES on the physiochemical, structural, and

techno-functional properties of DES-FBPI.

f) Evaluate the fundamental aspects of gelling properties and the molecular interaction of DES-FBPI in comparison to conventionally extracted (alkaline and salt extraction methods and commercial soybean protein as a standard) fava bean protein isolates.

Chapter 2

Literature Review

Novel Extraction Technologies for Developing Plant Protein Ingredients with Improved Functionality

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2. Literature review

2.1. Abstract

Conventional methods employed for protein extraction from different food materials have many drawbacks, including accelerated protein denaturation, production of huge wastewater, causing environmental hazards, high consumption of chemicals, high production cost, and low extraction yield. These limitations have created a major bottleneck for the food industries, particularly in the utilization of plant-based proteins, which have been reported to lose their techno-functional properties due to their extraction methods. To circumvent these limitations, innovative and green technologies such as enzyme-assisted extraction, deep eutectic solvent (DES) extraction, reverse micelles extraction, microwave-assisted extraction, ultrasonic-assisted extraction, and subcritical water extraction have emerged. These technologies offer advantages over conventional methods in terms of environmental safety, extraction efficiency, and enhanced techno-functional properties. However, the commercial adaptation of these innovative methods is still in its infancy. Therefore, understanding the mechanism of these extraction methods is crucial for their exploration and scale-up for industrial applications. This review provides an updated insight into the conventional and novel protein extraction methods and their challenges in the food industry. The advantages of these novel methods, particularly in terms of the techno-functional properties of plant-based proteins and their efficacy in developing value-added protein ingredients, are extensively discussed. Furthermore, the mode of action for the new and eco-friendly protein extraction methods is extensively discussed. Furthermore, the challenges and future perspectives of these methods are also reviewed.

2.2. Introduction

The global population is expected to grow by over 9 billion by the year 2050 (UN, 2017), and parallel to that, it has been estimated that world food production is required to increase by 70% (Hunter et al., 2017). Achieving this food production target is becoming more challenging since the traditional agricultural intensification methods are no longer considered eco-friendly (Bleakley & Hayes, 2017). Protein is a macronutrient with an unprecedented increase in demand in the future. In 2019, the global protein ingredient market generated USD 38.02 billion and was projected to have a compound annual growth rate (CAGR) of 9.1% from 2020 to 2027 (Protein Ingredients Market Analysis, 2019). In addition, it is projected that the global protein demand will surge to 943.5 million metric tons by 2054, indicating the need for alternative protein markets, including plant-based proteins (PIMA, 2017). This level of global protein demand necessitates technological capabilities and innovative research to meet protein production targets. From the Food Technologist's standpoint, recognizing the techno-functional properties of proteins and the impact of extraction methods is crucial in meeting the anticipated demand in the near future.

In addition to the nutritional and health benefits, food proteins directly impact product quality, which is associated with their specific functional properties (Elsohaimy et al., 2015). However, the functional properties of food proteins are influenced by several factors, including the interactions with water, proteins, lipids, carbohydrates, and other compounds, as well as environmental conditions such as temperature, pH, and ionic strength (Aguilar-Acosta et al., 2020). Additionally, the adopted extraction method has a significant impact on the techno-functional properties of the isolated protein (Ochoa-Rivas et al., 2017). Commonly used protein extraction methods include salt precipitation, solvent precipitation, and alkaline extraction; however, the latter has proven to be the most efficient method, as it renders proteins more soluble

by facilitating the efficient breakdown of hydrogen bonds (Cui et al., 2017). However, alteration of protein functionality during extraction due to accelerated protein denaturation, leading to changes/modifications in protein structure, is considered a drawback of the alkaline extraction method (Gao et al., 2020; Valenzuela et al., 2013). Therefore, it is imperative to develop novel extraction processes to produce protein ingredients with unique functionalities.

The higher consumer acceptability and concerns about sustainability within the general population have increased the attention toward plant proteins (Van Loo et al., 2017, Nadeeshani et al., 2022). As a result, animal proteins are gradually being replaced by various plant proteins in certain food formulations to produce novel functional and value-added products (Ismail et al., 2020). Besides human dietary applications, plant proteins are widely used in the pharmaceutical and pet food industries (PIMA, 2017). Moreover, plant proteins have shown promising potential as reliable sources of functional ingredients due to their unique techno-functional properties (Lonnie et al., 2020).

Currently, multiple protein extraction technologies such as alkaline extraction, isoelectric precipitation (Kalaydzhiev et al., 2020), salt-based extractions (Karaca et al., 2011a, 2011b), ultrafiltration/diafiltration (Hernández-Marín et al., 2019; Osemwota et al., 2021), and dry fractionation technologies (Schutyser et al., 2015) have been used individually and/or in combination for extracting plant proteins. However, these techniques have been associated with several limitations, including poor extraction yields, changes in protein techno-functional properties, low stability, reduced nutritional quality, undesirable color, and the generation of large amounts of wastewater, which may lead to environmental hazards (Valenzuela et al., 2013). Therefore, there is an urgent need to develop eco-friendly and novel extraction technologies to overcome the technological and scientific limitations of conventional protein extraction methods.

In this context, novel green extraction technologies include enzyme-assisted extraction (Naseri et al., 2020), deep eutectic solvent extraction (Yue et al., 2021), reverse micelles extraction (Sankaran et al., 2019), microwave-assisted extraction (Varghese & Pare, 2019), and ultrasonic-assisted extraction (Dabbour et al., 2018) have demonstrated promising results for extracting plants proteins with enhanced techno-functionality. In addition, these novel technologies are regarded as affordable, safe, and environmentally friendly; therefore, they could provide a clean label status (Pojić et al., 2018). However, these technologies have not been fully exploited in extracting plant protein. Thus, this review meets the necessity to assemble recent information on the status of novel and eco-friendly conventional protein extraction methods. Additionally, the challenges and future perspectives of these novel methods are extensively discussed.

2.3. Conventional protein extraction methods

The nature of the starting material is crucial for selecting the appropriate extraction method. The high amounts of polyphenols, lipids, and polysaccharides in plant tissues can interfere with protein separation and/or induce modifications in protein amino acid residues, posing significant challenges for plant protein extraction (Luís et al., 2016). Currently, the common method for removing interfering compounds relies on precipitation steps and phenol extraction, either individually or in combination (Silva-Sanchez et al., 2015). The various pre-treatment methods would potentially benefit in removing these interfering compounds before protein extraction. Hence, defatting, enzyme-assisted pre-treatment with cellulase and phytase enzymes, and/or phenolic extraction have been implemented to enhance the protein extraction yield and purity (Luangthongk am et al., 2015; Osemwota et al., 2021).

Several methods are used conventionally for extracting plant-based proteins. Among the various methods developed, alkaline extraction, isoelectric precipitation, dry fractionation, and the

use of inorganic and organic solvents have been employed extensively. In the alkaline extraction/isoelectric precipitation method, proteins are solubilized by increasing the pH beyond their isoelectric point (pI), rendering the protein molecules negatively charged and soluble in aqueous solutions. Following this, centrifugation is performed to remove insoluble materials, which are mostly non-protein molecules. Then, the solubilized proteins are precipitated by adjusting the pH of the extract to the pI, which imparts zero net charges on the protein (Tsermoula et al., 2019). The pI of most plant proteins ranges from pH 4.0 to 5.0, and complete solubilization can be achieved at pH 8.0-11.0. However, a higher protein extraction yield was recorded when pH >11.0 was used (Çelik et al., 2019; Gao et al., 2020). Gerzhova et al. (2016) reported the influence of the pH of the extracting media on the extractability of proteins from canola meal, where the extraction yields were 26.63% and 58.12% at pH 10 and 12, respectively, confirming solubility as a function of increasing pH.

Recently, techniques such as ultrafiltration/diafiltration have shown promising potential for increasing the extraction yield of proteins with improved functionality, particularly when combined with the alkaline extraction/isoelectric precipitation technique (Alonso-Miravalles et al., 2019; Jain et al., 2015; John & Sinha, 2019). In addition, these techniques inactivate protease and amylase inhibitors and reduce lectins and other anti-nutritional factors (Ali et al., 2010; Hadidi et al., 2020). Ultrafiltration/diafiltration is a separation process in which proteins solubilized after alkaline extraction are concentrated by removing water and low-molecular-weight non-protein compounds (Singhal et al., 2016). The molecular weight of the membrane commonly used for this method ranges between 1 and 100 kDa (Klupšaitė & Juodeikienė, 2015). A study conducted by Alonso-Miravalles et al. (2019) found that lentil protein concentrates prepared by ultrafiltration (10 kDa MWCO polysulfone membrane, 1:1.7 retentate: water ratio at 50 °C) had a higher protein

content (93.7%) than the isoelectric precipitation (85.1%), which in addition, resulted in higher dietary fiber, sodium, and phosphorus mineral contents.

The salt extraction technique is another widely used method for protein isolation, mainly from plant-based materials. This technique involves the salting-in and salting-out phenomenon (Boye et al., 2010b). In salting-in, the ionic strength of protein is increased via the addition of salt, which enhances protein hydration due to the increased interaction of the protein with water. Nevertheless, protein solubility has been reported to decrease rapidly at high salt concentrations (>1 M), which facilitates precipitation or "salting out" (Boye et al., 2010a). Ammonium sulfate and sodium chloride are the most commonly used salts in protein extraction (Sun & Arntfield, 2010). Stone et al. (2015) produced protein isolates from three pea cultivars using three extraction methods: alkali extraction/isoelectric precipitation (AE-IP), salt extraction dialysis (SE), and micellar precipitation (MP). Regardless of the cultivars, the protein extraction yield (% based on protein content) was in decreasing order: SE (68-72%) > AE-IP (62-67%) > MP (30-31%) (Stone et al., 2015). Furthermore, Karaca et al. (2011a) demonstrated the efficacy of salt precipitation in isolating proteins from several legumes compared with isoelectric precipitation. The authors reported that protein isolates produced from chickpeas (85.40% of proteins), fava bean (84.14%), lentils (81.90%), peas (88.76%), and soybean (87.59%) using isoelectric precipitation had higher protein yield than those extracted using salt extraction method, which ranged between 72 and 81%.

Dry fractionation is another method of protein extraction that has been used for plant protein extraction. This technology relies on the efficacy of milling to mechanically dissociate protein and other cellular compounds, including starch, based on particle size and density (Schutyser et al., 2015). The dissociated proteins can now be separated with the aid of an airstream based on their size (Assatory et al., 2019). Not leaving any chemical residues, minimum impact on techno-

functional properties, loss of insoluble protein, as well as low energy consumption are the primary advantages of the dry fractionation method (Schutyser et al., 2015). However, low protein extraction yield contributes to the limited application of the dry fractionation method (Schutyser et al., 2015; Singhal et al., 2016). The presence of other components, such as oil and fibers, and the loss of protein through coarse fraction are mainly responsible for the low extraction yield (Rempel et al., 2019). Pelgrom et al. (2013) studied dry milling ("impact milling" and "jet milling") in combination with air classification from yellow field peas (*Pisum sativum*). The results of this study indicated a decrease in protein yield from 75% to 39.9% and 86.5% to 15.5%, respectively, with increasing classifier speed from 2500 to 8000 rpm, but the protein content of jet and impact milled flours increased by 22.5 to 32.7% and 22.4 to 55.4%, respectively. However, optimum disentanglement of protein bodies and starch granules was observed in both impact and jet milling classification speed at 4000 rpm, and pea flour milled under this speed combined with air classification resulted in protein-rich concentrates (51%) at a maximum protein recovery rate of 76.8%. In another study, defatting and dry fractionation of soybean flour by a combination of impact milling and tribo-electrostatic separation showed that at an optimum classifier milling speed of 3000 rpm, soybean protein had higher integrity and compact structure (Xing et al., 2018). In another study, dry fractionation (with impact milling) and subsequent spontaneous solid-state fermentation (SSF) of chickpeas resulted in 28.4% of protein-enriched fractions and 47.7% of enriched starch fractions (Xing et al., 2020). Further, the protein-enriched fraction showed reduced phytic acid (17%) and indigestible α-galactosides (90%), along with improved techno-functional properties and nutritional value (Schutyser et al., 2015).

2.4. Challenges and limitations of current methods

Protein functionality is a critical factor in using proteins in food applications. Solubility, fat and water-binding capacity, foaming capacity and stability, emulsion capacity and stability, gelation, film-forming capacity, and viscosity are some of the key functionality traits (Boye et al., 2010a). Techno-functional properties of plant proteins are dependent on technological factors such as pH and ionic strength of the solvent, time, in addition to temperature, and particle size, which are parameters used in conventional protein extraction (Kolpakova et al., 2018). The extraction at extremely high temperatures and pH has resulted in protein isolates with poor nutritional and functional properties, mainly due to the excessive protein denaturation induced by heat and increased protein aggregation at higher pH values (Gao et al., 2020; Valenzuela et al., 2013). In addition, alkaline extraction methods have been linked with the formation of nephrotoxic lysinoalanine compounds via the conversion of amino acid residues such as cysteine and serine, which triggers the impairment in protein bioactivity (Zhang et al., 2018a).

The dark color of the extracted plant protein using conventional methods is another major limitation. The dark/dull color can be associated with the co-extraction of other compounds, mainly phenolic compounds. The use of sunflower meals is limited due to polyphenols impart a bitter taste, dark color, and astringency (González-Pérez & Vereijken, 2007). With the alkaline extraction, these polyphenols undergo oxidation, producing o-quinones or o-dihydroxy structures, which are highly reactive (Malik & Saini, 2017). Also, these o-quinones/o-dihydroxy compounds can link covalently with the thiol group (-SH) and amino group ($-NH_2$) of proteins at higher pH or create non-covalent hydrogen bonds at lower pH, imparting dark green to brown color to protein ingredients (Malik & Saini, 2017). Moreover, coextracted phenolic compounds can also negatively influence the functional properties of proteins. Subaşı et al. (2019) concluded that the polyphenols

negatively affected the foaming and interfacial properties of the isolate from defatted sunflower cake.

Apart from these structural and functional change-related issues associated with conventional extraction techniques, environmental safety is another major concern. For example, organic solvents, particularly *n*-hexane used in defatting raw materials, contribute to air pollution and are reported to be toxic (Kumar et al., 2017). Furthermore, sodium hydroxide and potassium hydroxide are used for the alkaline extraction process, and their neutralization generates huge amounts of wastewater with a high possibility of causing harm to flora and fauna (Zhang et al., 2015a). Therefore, new extraction technologies with less impact are required for environmental safety.

2.5. Recent advances in novel extraction methods

Green chemistry is considered a crucial step in scientific research for the sustainability of the environment. Extraction of the natural product under the label of green chemistry provides the opportunity to produce safer food ingredients, higher extraction yield, enhanced nutritional and functional properties, and clean labeling opportunities (Pojić et al., 2018; Wen et al., 2019). Furthermore, the novel protein extraction could further provide higher purity, less solvent usage, less energy consumption, and reduced effluent compared to conventional extraction methods (Chemat et al., 2019). The mechanism, advantages, and limitations of novel protein extraction technologies, including enzyme-assisted extraction (EAE), deep eutectic solvents (DESE), reverse micelles extraction (RME), subcritical water extraction (SWE), microwave-assisted extraction (MAE), and ultrasonic-assisted extraction (UAE), which are commonly labeled green protein extraction, particularly for plant-based proteins, are extensively discussed. Additionally, the

application of these extraction technologies over conventional protein extraction methods in terms of extraction yield and functional properties is presented in Table 2.1.

2.5.1. Enzyme-assisted extraction of protein (EA)

To separate proteins from plant cells, it is imperative to break down or hydrolyze other cell components, such as cellulose, hemicellulose, and pectin, using specific enzymes as illustrated in Figure 2.1 (Jung et al., 2006). Since enzymes are highly specific, other cell wall components are released with minimal impact on the properties of the isolated protein (Sierra et al., 2017). The yield and purity of the extracted protein using EAE solely depend on the degree of cell wall disruption. However, the degree of hydrolysis solely depends on the type and concentration of the enzyme, the ratio of enzyme and substrate, temperature, and time (Nadar et al., 2018). During cell wall degradation, endogenous proteases are liberated, which can initiate protein hydrolysis, thereby changing protein conformation and potentially producing short-chain peptides (Nadar et al., 2018; Pojić et al., 2018). Soybean pretreated with 2% of viscozyme L (enzyme complex composed of cellulase, hemicellulase, arabinase, β- gluconase, and xylanase) did not show any significant effect on protein content when used for 15 and 30 min as compared to the control. However, a significant increase in protein yield was observed when the enzyme concentration was increased to 6%. Furthermore, no significant effect was observed on the protein content during hydrolysis (Penha et al., 2020). de Figueiredo et al. (2018) demonstrated protein extraction from okara using a multi-enzyme complex (Viscozyme). The authors reported 56% protein content and 28% protein recovery under the optimum conditions of 53 °C, pH 6.2, and 4% enzyme concentration.

To further increase the efficacy of protein extraction, the combined application of EAE with alkaline or acid extraction has been explored. Sari et al. (2013) used oilseed meals such as

rapeseed, soybean, and microalgae to extract proteins using the EAE process combined with alkaline or acid extraction. The study revealed that protein extraction using acid extraction without EAE had a lower protein yield than the alkaline extraction, which was attributed to acid-reduced cell wall degradation. However, the extraction yield was further increased by adding enzymes (Protex 5L, Protex P, and Protex 40XL at 5% enzyme dosage and 3 hrs incubation period), particularly under alkaline conditions. In addition, EAE has been combined with other techniques, such as ultrasound, sonication, and microwave, to increase the extraction yield (Jiang et al., 2021; Nadar et al., 2018).

Several researchers have studied the impact of EAE on the techno-functional properties of plant proteins. Cell wall degrading enzymes, including multicomplex or single enzymes, have been widely used to recover high-quality proteins from plant matrices with improved functional properties (Kumar et al., 2021). Perović et al. (2020) reported a significant increase in solubility, foam capacity, and foam stability of proteins recovered from soybean grit with enzymatic pretreatment (1 hrs at pH 5.5 using an enzyme mix of cellulase, pectinase, and xylanase) followed by alkaline extraction at pH 8.0 and 50 °C, compared to alkaline only extracted protein (3 hrs at pH 8.0 and 50 °C). The improved techno-functional properties were attributed to the reduced extraction time in the combined EAE and alkaline treatment, which prevented the isolated protein from autolysis compared to alkaline extraction alone. Similarly, improved water holding capacity, foaming capacity, foam stability, and emulsion stability index were documented by Jiang et al. (2021) for *Akebia trifoliata* (Thunb.) Koidz seed protein isolates extracted by combining cellulase (0.1 g of 10 U/mg) and alkaline treatment compared to the control without enzyme. In another study by Wei et al. (2018), the protein extracted from soybean by EAE with a cascade of enzymes (β-glucosidase, phytase, and acid-protease) exhibited higher thermal stability and surface

hydrophobicity than that of alkaline or acid-extracted proteins. Therefore, EAE shows promising potential for extracting plant proteins with improved techno-functional properties. However, the cost and activity of some of the enzymes are some of the concerns that need to be addressed for increased commercial applications of EAE in the plant-based protein industry.

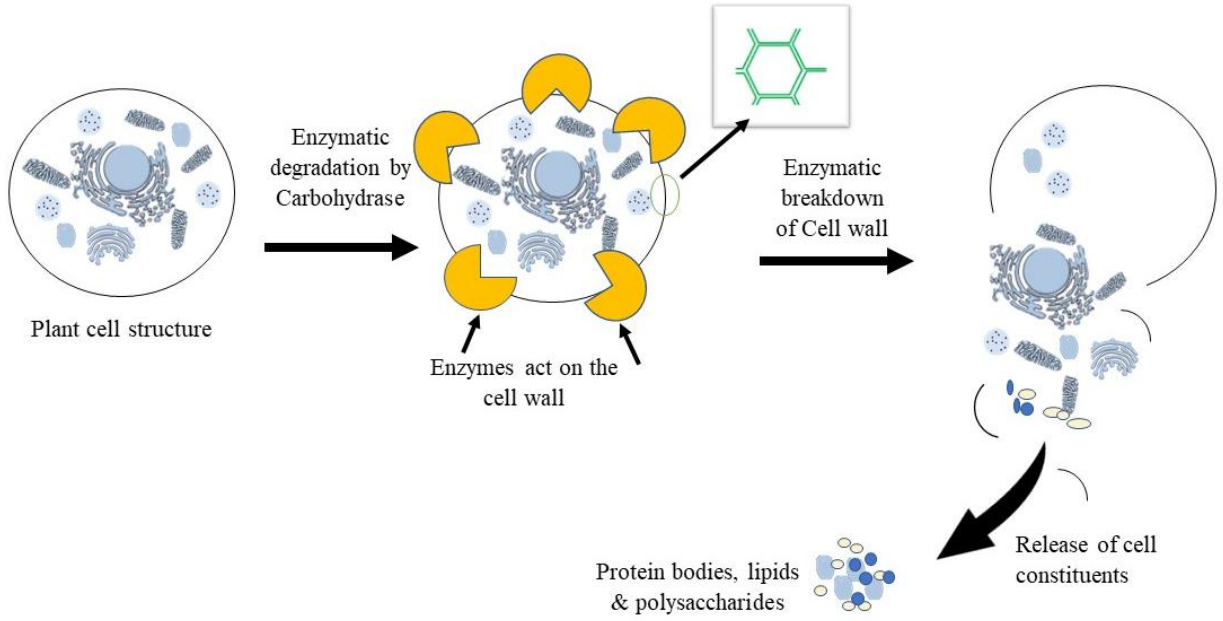

Figure 2. 1. Schematic diagram of the enzymatic breakdown of the plant cell wall during enzymatic-assisted protein extraction

2.5.2. Deep eutectic solvent (DES) extraction

Deep eutectic solvents (DES) are recognized as a class of ionic liquid (IL) analogs because of the similarities in their characteristics and properties compared to ILs (Smith et al., 2014). However, the unique chemical properties of DES make it suitable for application in food and metal processing applications (Chen et al., 2019; Smith et al., 2014). DES is formed from the eutectic mixture of Lewis or Brønsted acids and bases (Smith et al., 2014). Generally, DES is obtained from the complexation of quaternary ammonium salts such as N,N-diethyl-2-hydroxy ethanamidium chloride, tetramethylammonium chloride, choline chloride, tetrabutylphosphonium

bromide, methyltriphenylphosphorium bromide, tetrapropylammonium bromide, etc., otherwise known as hydrogen bond acceptor (HBA) with a hydrogen bond donor (HBD) such as glycerol, sorbitol, thiourea, acetamide, benzamide, imidazole, malonic acid, and urea, etc. Figure 2.2 illustrates the DES formation mechanism and structure (Tomé et al., 2018; Xu et al., 2015), where the resulting mixture contains non-symmetric and large ions with low lattice energy and low melting points (Smith et al., 2014). Abbott et al. (2005) heated a range of quaternary ammonium salts with $ZnCl_2$ and measured the freezing points of the resulting liquids. The lowest melting point of 23–25 °C was obtained when choline chloride was used as the ammonium salt. Since this initial study, a range of eutectic mixtures of salts and HBDs have been developed for different applications (Smith et al., 2014).

The increasing demands for eco-friendly processes within the framework of green and sustainable chemistry and the recognition of the advantageous properties of DES have positioned the use of DES as an alternative to conventional organic solvents in diverse fields (Tomé et al., 2018). Conventional organic solvents, such as methanol, acetonitrile, ethanol, hexane, acetones, etc., have been used in food processing technologies, including extraction, separation, and pre-concentration. Although these conventional organic solvents are low-cost and easy to process, potential environmental pollution is a significant concern (Chen et al., 2019; Puranik et al., 2009). DES has been explored as a sustainable, green processing technology for extracting different food components to circumvent these limitations. As eco-friendly or green solvents, DESs have attracted increasing interest in the scientific community. Owing to its low cost, biodegradability, and low toxicity, the number of new DESs has increased considerably (Li & Row, 2016). Besides, the synthesis of DES is 100% atom economical, easy to handle, and requires no purification, thus making its large-scale use feasible (Zhang et al., 2012). Due to the excellent solvent properties of

DES, it has been widely used in electrochemistry, organic synthesis, biocatalysis, biodiesel preparation, metal-catalyzed or metal-mediated organic reactions, and extractions (Cui et al., 2018). DES's ability to dissolve both polar and nonpolar metabolites (Paiva et al., 2014) has gained more attention as a green extraction solvent for food components such as polyphenols (Chemat et al., 2019; Cui et al., 2018).

The application of DES in protein extraction and purification has been demonstrated at a limited capacity in recent studies. DES-based aqueous two-phase systems (ATPs) have shown promising potential in protein extraction and purification (Iqbal et al., 2016). ATPs are generally formed by combining two non-consolute polymers or polymers and an inorganic salt solution (Atefi et al., 2016). These phase components were mixed above a specific critical concentration and separated into two unambiguous aqueous phases. Thus, the principal mechanism for protein extraction is associated with increased ionic strength. The efficacy of ATPs in protein extraction or purification, when compared to other methods, is associated with their high water content and low interfacial tension, which prevented protein denaturation or loss of biological activity (Li et al., 2016). Li et al. (2016) established betaine–urea DES-ATPs for the extraction of model proteins, in which the protein extraction efficiency of up to 99.82% with no conformational changes under the optimum conditions (salt concentration-0.75 g/mL, DES-1.4 g, separation time- 12 min, amount of protein-15 mg, and temperature-30 °C). The enhanced extraction of proteins from rapeseed cake (RC) and evening primrose cake (EPC) using a glycerol-choline chloride DES was demonstrated by Grudniewska et al. (2018)The extraction yield after precipitating the protein-rich DES extract by adding water (antisolvent) was dependent on the extraction temperature, which reached a maximum of 20% and 35% at 140 °C from RC and EPC, respectively, with a protein content of 40–50%.

The type of HBD and HBA used significantly affects the protein extraction efficacy of the resultant DES system. In the work of Wahlström et al. (2017), the protein was extracted from Brewer's spent grain (BSG) using novel carboxylate salt (NaAcO)- urea-based DES, and choline chloride: urea-based DES was investigated. NaAcO-urea-based ATPs were a more suitable protein extractant than choline chloride: urea-based DES, in which up to 79% extraction yields from BSG were obtained. This was due to the dissolution of proteins insoluble by nature or denatured during the brewing process (Wahlström et al., 2017). The extraction of BSA using DES-ATPs based on ChCl–urea, tetramethylammonium chloride (TMACl)–urea, tetrapropylammonium bromide (TPMBr)–urea as well as ChCl–methylurea was demonstrated, out of which ChCl–urea-based DES showed the highest efficacy (Zeng et al., 2014). The hydrophobic interactions, hydrogen bonds, and the salting-out effect played an important role in the transfer process of proteins into the solution. Nevertheless, the amount of DES, the concentration of K_2HPO_4, separating time, and temperature significantly influenced the protein extraction yield of ChCl–urea-based DES when used for extracting BSA (Zeng et al., 2014). Zhang et al. (2016) demonstrated the efficacy of ternary and binary DES as novel extraction media for protein partitioning. Four types of binary DESs, which are composed of tetramethylammonium chloride (TMAC) as the HBA and HBD (urea, glycerol, ethylene glycol, D-(+)-glucose), and four kinds of ternary DESs comprising TMAC and an HBD (glycerol) combined with urea, ethylene glycol, D- (+)-glucose, D-sorbitol was investigated. The TMAC-urea (TMAC-U) and TMAC-glycerol-urea (TMAC-G-U) had the promising potential for protein partitioning (Zhang et al., 2016). Both DES-ATPs systems showed high efficacy for BSA partitioning, in which 99.31% and 98.95% were obtained for TMAC-U and TMAC-G-U, respectively, using the optimized condition of 1.52 g of DES, 075 g/mL salt concentration, 10 mg BSA, and temperature of 25 °C (Zhang et al., 2016).

Combining DES with other technologies has been demonstrated to increase protein extraction efficacy further. Liu et al. (2017) demonstrated the extraction of pumpkin seed protein with poly (ethylene glycol)-based DES under ultrasound-microwave synergistic extraction conditions. The highest protein yield (93.95%) was recorded for aqueous poly (ethylene glycol) (PEG 200)-based DES using this optimized condition PEG 200- based DES concentration, 28% w/w; solid to liquid ratio, 28 g/mL microwave power, 140 W; and extraction temperature, 43 °C (Liu et al., 2017). However, this technology has not been fully exploited in protein extraction and ingredient development, particularly in plant-based proteins, creating a knowledge gap on the optimized DES protein extraction conditions for different plant sources. Also, the recovery of DES after protein extraction poses a serious challenge for its industrial scale.

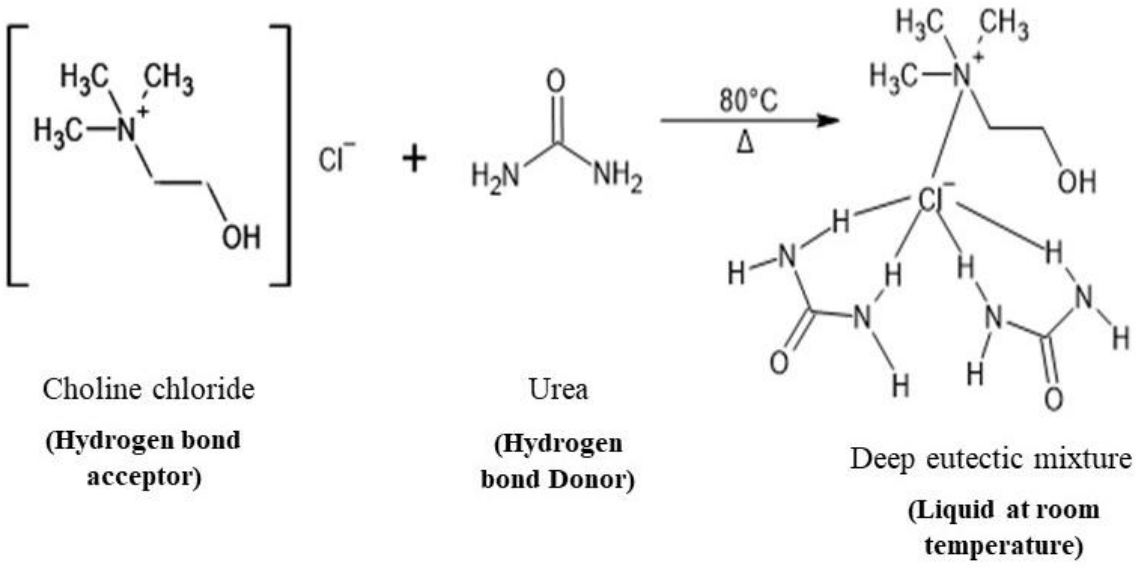

Figure 2. 2. Formation of the deep eutectic solvent mixture using choline chloride and urea

2.5.3. Reverse micelles extraction

Reverse micelles (RM) can be defined as "nano-sized spherical enclosures, which consist of an inner water pool core and an outer surfactant molecule-based organic solvent" (Sankaran et al., 2019). The surfactants consist of a polar head group oriented toward the micelle's inner aqueous core, whereas a lipid-loving hydrocarbon tail is oriented towards the organic solvent (Chia et al., 2019). Micelles allow the solubilization of targeted biomolecules inside the inner water core (Du et al., 2020b); hence, extraction of the targeted biomolecule can be performed successfully (Figure 2.3).

Due to different water content within the RM, inner core water is physiochemically different from the bulk water. RM anchors the biomolecule to be solubilized inside the core without causing conformational changes (Mohd-Setapar et al., 2014). This unique characteristic of RM has been applied in protein extraction and purification (Wong et al., 2018). There are two steps involved in the extraction of protein by RM, which are forward extraction (FE) and backward extraction (BE) (Gangadharappa et al., 2017). FE involves the solubilization of proteins within the inner aqueous core in three steps of RM formulation, protein encapsulation in RM, and phase separation; soluble proteins within the water core are then recovered from the backward process (Liu et al., 2008).

The extraction of protein using the RM technique is influenced by a range of parameters, including water content, pH, ionic strength, temperature, type of surfactant and its concentration, and type of solvent (Pawar et al., 2017). Furthermore, RM properties, such as size, shape, and structure, depend on the solvent used and affect the amount of biomolecules transferred (Pawar et al., 2017). In food-related applications, ionic surfactants such as sodium salt of sulphosuccinic acid bis(2-ethylhexyl) ester (AOT) are commonly used (Zhao et al., 2018; Zhao et al., 2019). The electrostatic interaction between ionic surfactant molecules and protein molecules is the major

principle involved in FE. Therefore, pH and ionic strength, which are responsible for protein charges, are the key factors for the RM extraction process (Sy Mohamad et al., 2020).

Factors affecting the efficiency of the forward and backward protein extraction protocols have been well-documented in the literature. Zhang et al. (2021a) investigated the optimum conditions for extracting soybean proteins using RM forward and backward methods. The authors found the highest extraction efficacy for an extraction time of 25 min, pH of 3.0, the temperature of 45 °C, KCl concentration of 0.0 mol/ L when forward extraction was used, and extraction time of 10 min, pH of 4.0, the temperature of 30 °C, KCl concentration of 0.5 mol /L was used for the backward extraction. Furthermore, Guo et al. (2015) investigated the effect of several kinetic factors (ion temperature, strength, and pH) during RM backward extraction to separate peanut protein from full-fat peanut powder using response surface methodology. The authors documented an optimum condition of 7.5, 1.1 mol/L, and 35 °C for pH, ion concentration, and temperature, respectively, in which 79.03% of protein was recovered. Similarly, Reis et al. (2016) documented that stirring time, temperature, molar ratio, and sample size used in RM influence the protein recovery from jackfruit flour. The authors reported optimum conditions of 25 °C, 120 min, 100 mg, and 50 for temperature, stirring time, the mass of flour, and H_2O/SDS ratio, respectively, in which a protein extraction yield of 79% was reported. In another study by Zhang et al. (2017b), RM forward extraction yield of grape seed protein was able to achieve up to 82.3% under optimum conditions of 39 mmol/L cetyl-trimethyl-ammonium bromide (CTAB), pH 5.6, 0.01 mol/L NaCl concentration, and 2.1 mg/mL crude protein concentration.

The application of new protein ingredients processed through RM depends on the techno-functional properties and conformational changes. Zhang et al. (2021a) documented varying emulsifying properties in emulsion formation and stability for soybean protein extracted using RM

as a function of the type of extraction (backward or forward), time, pH, and temperature, which was attributed to the structural changes in the protein. Additionally, soybean protein extracted using the RM method revealed improved solubility, oil absorption capacity, foaming capacity, foaming stability, emulsifying capacity, and emulsifying stability compared to the conventional alkaline extraction method (Zhao et al., 2018). Nutritional properties were also improved with RM, measured by total amino acid content, amino acid score, and protein efficiency ratio. Yao et al. (2021) compared the gelling properties of soybean 11S globulin extracted using the RM extraction method to those of alkaline extraction. The authors reported higher loss and storage modulus for the gel formed by soybean globulin extracted using RM compared to alkaline extraction, which was attributed to the stronger heat resistance and higher denaturation temperature of the soybean 11S globulin.

Combined treatment with microwave-assisted extraction has been documented to further increase the efficacy of RM extraction from plant materials. In the study of Wang et al. (2021) the process parameters for isolating walnut protein using RM combined with microwave extraction were optimized using response surface methodology as 30 min, 45 °C, and 3:1 (v/v) for extraction time, temperature, and the ratio of the aqueous solution to forward extraction solution, respectively, in which the extraction yield of 95% was achieved. Similarly, the techno-functional properties, including solubility, water-holding capacity, oil-holding capacity, foaming, and emulsifying properties of the walnut protein extracted using the combined method, were higher than those extracted using RM only (Wang et al., 2021).

Conformational changes in proteins extracted using RM extraction have been documented. Changes in protein conformation can increase and/or decrease the surface hydrophobicity of proteins, thereby influencing their solubility and interfacial properties, and consequently, their

techno-functional properties (Fadimu et al., 2022). According to Yao et al. (2021), soybean 11S globulin extracted using RM had a lower β-sheet structure content and higher β-turn structure content compared to that extracted using alkaline extraction. The protein unfolding and surface hydrophobicity were also lower in RM-extracted soybean 11S globulin compared to those extracted using alkaline extraction (Yao et al., 2021). X-ray diffraction (XRD) and scanning electron microscopy (SEM) analysis also revealed the increased β-sheets with augmented pore structure in soybean protein powders obtained by RM extraction (Zhao et al., 2015). Moreover, walnut protein fractions obtained from RM significantly increased the sulfhydryl content in globulin, prolamin, and glutelin proteins compared to the conventional method (Zhao et al., 2019). Furthermore, FTIR analysis revealed higher proportions of α-helix in globulin and β-sheet structure in prolamin compared to conventionally extracted protein (Zhao et al., 2019). Despite these findings, the underlying mechanism of structural changes of protein by RM has not yet been fully elucidated. Therefore, future research is needed to understand the mechanism better and scale up the process for new food applications.

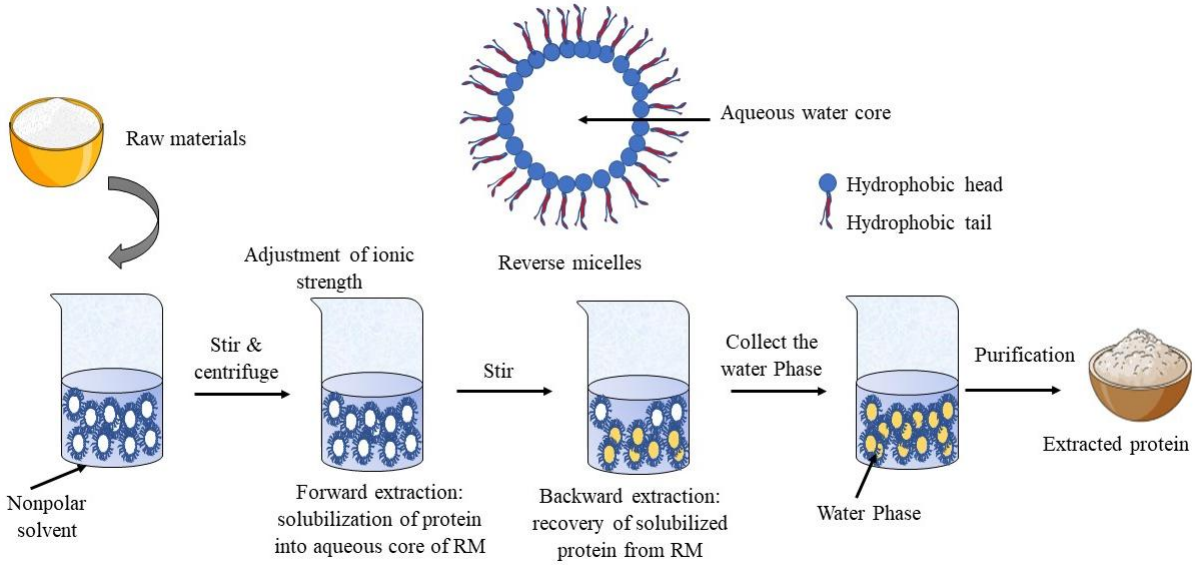

Figure 2. 3. Schematic illustration of reverse micelles

2.5.4. Microwave-assisted extraction (MAE)

Recently, MAE has gained attention due to its unique heating system, moderate cost, and good performance under atmospheric conditions (Pimentel-Moral et al., 2018). First, the transfer of energy by an electric field to the material is based on the dipole polarization, in which molecules are aligned with the electric field, holding a dipole moment that produces the molecular friction, liberating energy to the medium, and then with the subsequent conduction process (Adetunji et al., 2017). Secondly, when electromagnetic radiation is applied, ionic conduction induces the movement of charged ions inside the solvent by generating resistance within the solution, which results in friction and, consequently, heat (Ekezie et al., 2017) (Figure 2.4). When microwave heat approaches the moisture inside the cell matrix, evaporation and intense pressure build up in the cell wall (Ekezie et al., 2017). This will rupture the cell wall and cause the release of active constituents via the breakdown of hydrogen bonds, which enables the migration of dissolved ions, increased porosity of the biological matrix, and extraction of compounds of interest (Kadam et al., 2013; Kapoore et al., 2018; Pojić et al., 2018). The efficacy of MAE is influenced by several factors, such as temperature, solid-to-liquid ratio, extraction duration, microwave power, nature of samples, time, and stirring effect (Chan et al., 2011).

Protein extraction from different sources using MAE has been demonstrated. In a recent study by Chew et al. (2019), MAE three-phase partitioning method was developed as an effective green protein extraction method for *Chlorella vulgaris* microalgae species. A recovery yield of 63.2% with a separation efficiency of 67.2% was obtained for the optimized condition as follows: 30% ammonium sulphate, 1:1 ratio of slurry to t-butanol, 120 sec microwave time, 80% microwave duty cycle, 100 W microwave power, and 0.5 % (w/w) microalgae biomass

concentration. Furthermore, the amount of protein extracted from *Jackiopsis ornata* roots by MAE under the optimized condition of 30:1 (v:w) liquid-to-solid ratio, 0.022 radius particle size, 65 °C temperature, 300 W microwave power, and 20 min extraction time was the highest, in which 20.42% protein yield was obtained (Elhag et al., 2019). Similarly, the processing parameters for MAE, including pH 7–10, 30 sec–8 min extraction time, 1:10–1:40 solid to solvent ratio, and 30 - 70 W microwave power, were investigated for protein isolation from watermelon seed Behere et al. (2021). The authors reported that 50 W, 1:30, 10, and 2 min as microwave power, solid-to-solvent ratio, pH, and time, respectively, were the optimized conditions for extracting protein from watermelon seed using MAE, in which the recovery rate of 90% was higher than the 72% for conventional and 87% for ultrasound-assisted methods. Similarly, the recovery rate for protein extracted from coconut using MAE (4.31 kW/kg by pulse, 3 pulses of 20 sec, 1 min, 2.5 GHz) was higher than that extracted using conventional pH adjustment methods (Martínez-Padilla et al., 2022).

The optimization of protein extraction from coffee silverskin, a by-product obtained from coffee fruit processing, was demonstrated by Wen et al. (2021) using MAE for different times (10 and 20 min at 434.7 W. The highest protein recovery rate of 43% was achieved for MAE at 10 min with 0.6 M NaOH, compared to other extraction methods, including ultrasound-assisted and alkaline-acid extraction. Phongthai et al. (2016) optimized the protein extraction process from rice bran using MAE. The extracted protein yield using the optimized conditions of 1000 W microwave power, 90 sec extraction time, and a solid-to-liquid ratio of 0.89 g rice bran/10 mL was increased by 1.54-fold compared to the traditional alkaline extraction method. MAE yielded higher (75.9%) rice bran protein content compared to 71.7% for traditional alkaline extraction, and with a significant reduction in the extraction time (Bedin et al., 2020). Furthermore, Ochoa-Rivas et al.

(2017) documented the effect of MAE on the protein yield extracted from peanuts compared to the traditional alkaline extraction. The protein recovered using the optimized condition of MAE (725 W for 8 min) was 55% with 100% purity, which is a 77% protein increase compared to the conventional extraction.

The combination of MAE with other methods has also been documented as a potential solution to increase protein recovery rates from plant sources. For instance, Roslan et al. (2022) demonstrated the efficacy of MAE with salt precipitation for extracting soluble hydrolysate protein from a mixture of defatted soybean meal and jackfruit peel. The authors reported 8.7 min, 300 W, and 0.084 M as the optimized conditions for protein extraction from the biomass cocktail, achieving a 75% recovery rate. Combining MAE with enzymatic extraction has been shown to increase protein extraction yield further. In the recent work of Görgüç et al. (2020), the combined MAE and enzymatic extraction for recovering protein from sesame bran was investigated. Under optimal conditions (51 °C and 29 min), MAE alone increased the protein yield from 24.5 to 62.3%, compared to the alkaline extraction method. However, when MAE was combined with enzymatic extraction using Alcalase, the protein yield increased by 91.7% under the optimum conditions of 1.94 AU/100 g enzyme concentration, 49 °C, and 98 min extraction time. Based on scanning electron microscopy, the images revealed that MAE caused more irregular fragments and cell wall ruptures, whereas alkaline extraction revealed porosity and a slightly swollen structure. Furthermore, SEM images revealed more disordered structures with excess cell wall damage when MAE was combined with enzymatic extraction. This suggests that the morphological changes observed through the MAE and enzymatic treatments indicate effective liberation of bound protein molecules, as shown by the alterations in pore structures (Görgüç et al., 2020).

MAE's impact on the techno-functional properties of the extracted protein has been documented. Ochoa-Rivas et al. (2017) found that peanut protein extracted using MAE exhibited improved water absorption, foaming capacity, emulsifying activity, and in vitro digestibility compared to conventional extraction. However, a lower nitrogen solubility index and foam stability were recorded for MAE peanut protein compared to the control (Ochoa-Rivas et al., 2017). This was attributed to the changes in the tertiary structure induced by the thermal treatment, which was evident by the increased β-sheet content and reduced α-helix content in the MAE peanut protein isolate. Furthermore, the molecular vibration and cavitation enhanced by the microwave might influence molecular aggregation and modify the functional properties. In another study by Varghese and Pare (2019), a significant increase in protein solubility in soybean milk was obtained from soybean subjected to the MAE process using the optimal condition of 675 W, 80 °C, and a stirring speed of 160 rpm. This was associated with the reduced protein denaturation in MAE-assisted protein isolate (Varghese & Pare, 2019). Phongthai et al. (2016) reported that the protein extracted from rice bran using MAE (1000 W, 90 sec time, and a solid-to-liquid ratio of 0.89 g rice bran/10 mL of distilled water) with subsequent alcalase hydrolysis had the highest solubility when compared to protein obtained by MAE and the conventional method. The high solubility of products from MAE, combined with enzymatic hydrolysis, was associated with reductions in the molecular weight of the protein, as well as greater surface exposure of hydrophilic amino acids (Phongthai et al., 2016). In addition, the water-holding capacity, oil-holding capacity, and foaming capacity were higher in watermelon seed protein extracted using MAE compared to conventional methods (Behere et al., 2021). Also, the functional properties and digestibility of protein extracted from cold-pressed sesame seed meal were enhanced when MAE extraction was employed (Sá et al., 2022). Therefore, MAE has demonstrated the efficacy of isolating proteins with superior

techno-functional properties and digestibility without compromising the extraction yield and protein purity. The major limitation in the industrial-scale up of MAE for plant-based protein is the thermal effect induced by the inverse heat transfer of microwave irradiation, which can lead to undesirable structural modifications.

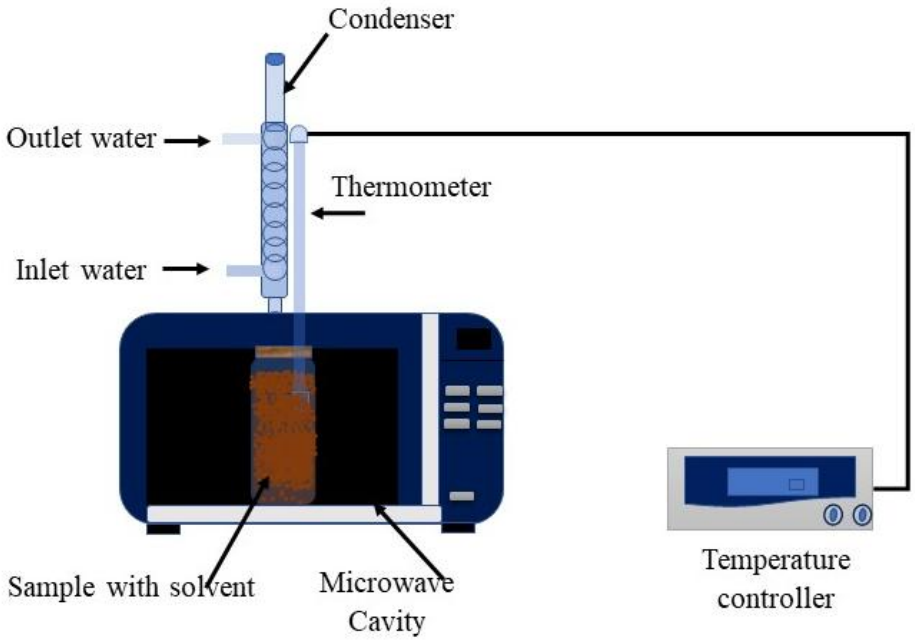

Figure 2. 4. Schematic illustration of microwave-assisted protein extraction

2.5.5. Ultrasonic-assisted extraction (UAE)

UAE process has gained researchers' attention in extracting compounds from plant and animal sources (Fadimu et al., 2022; Mason et al., 2015). UAE is a physical extraction process that enables the rapid and more effective extraction of target compounds from food materials via the rupture of cells using ultrasound waves (Fadimu et al., 2022). Ultrasound waves (UW) with a frequency above 20 kHz carry high energy as they travel through a medium (Fadimu et al., 2022). Alternatively, UW compresses and stretches during its movement in a medium, thereby forming localized negative pressure buildup. When the stretching phase, or “rarefaction,” occurs, the

generated pressure is sufficient to overcome intermolecular binding forces, resulting in bubbles or tiny cavities within the medium. This phenomenon is termed “cavitation” (Picó, 2013). These bubbles or cavities grow bigger with successive cycles and later collapse violently; thus, a massive amount of energy is released into the system. When the bubbles collapse, the localized pressure can be increased to more than 400 MPa, which can disrupt cell membranes to facilitate the movement of solvent in and out of the cell, as well as the liberation of target compounds (Fadimu et al., 2022; Flint & Suslick, 1991) (Figure 2.5).

Additionally, the cavitation generates micro-turbulence and high-velocity inter-particle collisions, accelerating cell diffusion and enhancing mass transfer across cell membranes (Picó, 2013). Consequently, disruption of biological cell walls will eventually lead to the release of cell contents (Awad et al., 2012). The efficacy of UAE in extracting protein depends on several factors, including the ultrasonic power, extraction time, the ratio of liquid to solid, and extraction temperature. Therefore, these parameters must be optimized to develop ideal extraction conditions for each material.

The extraction of plant protein using UAE has been demonstrated. In the study by Martínez-Padilla et al. (2022), the recovery rate of coconut protein extracted using UAE (24 kHz; 2.5 min; 6.85 W/cm^2, 0.573 kW/kg) was compared to those extracted using MAE (4.31 kW/kg by pulse, 3 pulses of 20 sec, 1 min, 2.5 GHz) and conventional method (pH adjustment). The authors reported a higher protein recovery rate for UAE, followed by MAE, and lastly, the conventional method. Gadalkar and Rathod (2020) reported that the optimum extraction conditions for watermelon protein using UAE were 9, 1:50 w/v, 30 °C, 90 W, 25 kHz, and 75% for pH, powder to solvent ratio, temperature, ultrasound power, frequency, and duty cycle, respectively, in which the 87% extraction rate was obtained. In the study of Zhu and Fu (2012), the protein extracted from defatted

perilla seed meal using UAE under the optimized condition (extraction temperature of 40 °C, power of 61 W, extraction time of 12 min, and ratio of liquid to solid of 40 mL/g) was higher than the conventional methods. YuCetepe et al. (2018) recovered 29.05% protein from blue-green microalgae S*pirulina platensis* using UAE under the optimized conditions of 45 °C, pH 7.46, and 120 min. The UAE recorded an increased protein yield in extracting protein from rapeseed meals when compared to the traditional alkaline method (Dong et al., 2011b). The optimized UAE conditions (450 W ultrasonic power, 84 min ultrasonic treatment time, 1:24 solid: liquid ratio, pH 11.5, and 35 °C ultrasonic temperature) recovered 76.83% of the protein, whereas the traditional method recovered 35.43% (Dong et al., 2011b). Similarly, Guzmán-Lorite et al. (2022) demonstrated the efficacy and optimized the processing parameters of UAE (0.5–15 min time, and 20–100% wave amplitude) in recovering proteins from pomegranate seed waste (PSW). The authors also compared the recovery rate of UAE to that of pressurized liquid extraction (PLE). It was reported that the optimized conditions for protein extraction from PSW were an extraction time of 5 min and an amplitude of 20%, during which 50% of the protein was recovered. Although a higher protein recovery rate was reported for PLE, the shorter processing time and lower amount of coextracted polyphenols gave UAE the leverage over PLE.

The combination of UAE and other methods has been shown to increase the efficacy of plant protein extraction. For example, the protein recovery rate was higher when protein extraction from coffee silverskin was done using UAE (100% ultrasound amplitude, 38.0 W/cm^2 ultrasound intensity, and time of 10 min) in combination with different concentrations of NaOH (0.2 and 0.6 M) when compared to those extracted without sonication (Wen et al., 2021). Furthermore, Silva et al. (2022) demonstrated protein extraction from Spirulina (*Arthrospira platensis*) using UAE (25 Hz at 30 ℃ for 45 min), followed by isoelectric precipitation, in which the extraction yield and

protein yield were recorded at 40.0% and 43.6%, respectively. Similarly, Lafarga et al. (2018) demonstrated the efficacy of UAE alone or combined with alkaline extraction for protein isolation from Ganxet beans (*Phaseolus vulgaris* L.). The authors found that protein recovery increased when the beans were subjected to a 0.4 M NaOH treatment followed by UAE (40 kHz and 250 W for 60 min) compared to those extracted with UAE or alkaline treatment alone.

Apart from yield improvement, UAE treatment has been reported to improve the digestibility of plant proteins via protein modification. Varying degrees (87.45 to 97.81%) of protein digestibility were recorded for protein extracted from blue-green microalgae *Spirulina platensis* using UAE under different conditions (pH: 7.0- 9.0, temperatures: 25- 45 °C, and time:60 -120 min), in which the highest was recorded when 45 °C, pH 7.46, and 120 min UAE condition was used (YuCetepe et al., 2018). Similarly, Gadalkar and Rathod (2020) reported a higher digestibility of 89% for protein extracted from watermelon seed using UAE compared to 80% and 62% for MAE and conventional methods, respectively (Behere et al., 2021). Furthermore, Li et al. (2013) documented an increase in digestibility for peanut protein when subjected to a sequential ultrasonication process. Solubility, enhanced cleavage of peptide bonds, loosened peanut kernel structure, and significantly lowered Immunoglobin E (IgE) binding of peanut extract were also reported for peanut protein subjected to the UAE when compared to the untreated counterpart (Li et al., 2013). Pan et al. (2020) reported an improvement in rapeseed napin digestibility after it was subjected to UAE. According to the secondary structure analysis through FTIR, the results indicated that protein disulfide bonds (S-S) were broken by the high shear energy waves generated during ultrasound treatment, leading to improved digestibility of napin (Pan et al., 2020). Similar findings were reported for proteins extracted from rice bran and subjected to UAE under conditions of 20 kHz, 600 W, 50 °C, and 60 min (Zhang et al., 2018b). The marked increase in S-H and S-S

content of rice proteins extracted using UAE compared to those extracted without UAE was attributed to changes in protein structure and the exposure of buried groups during protein unfolding, as well as the exposure of hydrophilic groups, which led to increased solubility (Zhang et al., 2018b).

The impact of the UAE on the techno-functional properties of plant-based protein has also been documented. Protein extracted from watermelon seed using UAE had higher water holding capacity, oil holding capacity, emulsifying capacity, and stability than that extracted using conventional methods (Gadalkar & Rathod, 2020). Similarly, the functional properties and protein digestibility of protein extracted from cold-pressed sesame seed meal were improved when UAE was used as compared to conventional methods (Sá et al., 2022). Therefore, the UAE demonstrated promising efficacy in enhancing the yield, functionalities, and digestibility of the extracted plant proteins. Nevertheless, the application of the UAE for plant protein extraction and modification is still in its early stages. In addition to the high installation cost, the undesired structural modification induced by ultrasound also contributes to its industrialization challenges.

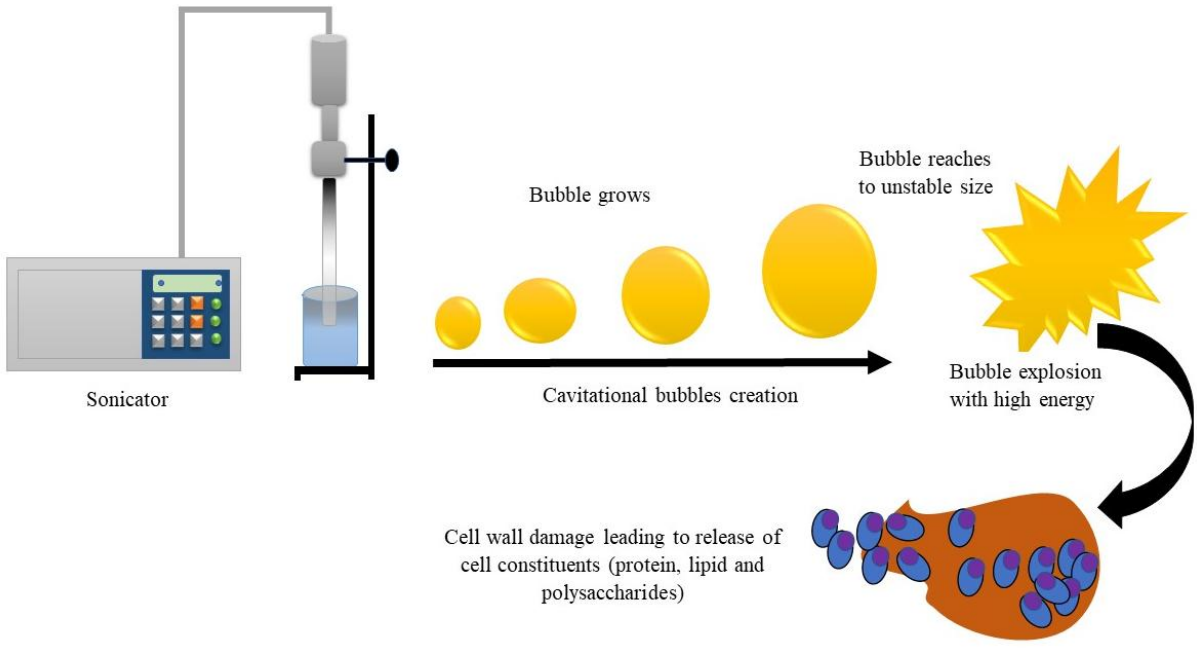

Figure 2. 5. Schematic diagram of ultrasound-assisted protein extraction's principle.

2.5.6. Subcritical water extraction (SWE)

SWE technology is one of the recent advancements in extracting compounds from agricultural biomass. SWE technology offers lower production costs, is chemical-free, and has shorter production times than conventional methods. Subcritical water is defined as water that is kept under sufficient pressure (1–22.1 MPa) to maintain its liquid state at a temperature between the boiling point (100 °C) and the critical point (374 °C) of water (Ju & Howard, 2005). Hydrogen bonds in water are disrupted at higher temperatures and pressure, decreasing its viscosity and surface tension, but the increased diffusivity allows better penetration into the matrix particles (Zhang et al., 2020). To keep water in a liquid state at temperatures higher than the boiling point, the application of sufficient pressure is essential, and high pressure further promotes the dissolution of analytes through the pores of the matrix (Wijngaard et al., 2012). Figure 2.6 illustrates the mechanism of subcritical water-assisted protein extraction

Solute-matrix and solute-solute interactions can be disrupted by the energy supplied by subcritical water, thereby reducing the activation energy required for the desorption process. Additionally, elevated pressure can assist water movement into the matrix (Teo et al., 2010). Higher temperatures result in a lower polarity of subcritical water, allowing it to separate compounds into polar, medium-polar, low-polar, and non-polar categories. Several factors, including temperature (Hawthorne et al., 2000), solvent rate (Plaza & Turner, 2015), particle size (Wiboonsirikul & Adachi, 2008), extraction time (Zhao et al., 2019), and solvent to sample ratio (Ravber et al., 2015) have been known to influence the efficacy of SWE in extracting compounds.

The extraction of plant proteins using SWE has previously been demonstrated. Švarc-Gajić et al. (2020) reported the efficacy of SWE for extracting protein from pumpkin, flax, and hemp

seed cakes under different conditions (N_2, CO_2, and N_2 + catalyst) at 2 MPa, in which the extraction efficacy was highest with SWE in CO_2 atmosphere, followed by SWE in N_2 + catalyst atmosphere. The yield and purity of proteins extracted from brewer's spent grain, pasture grass, and cyanobacteria (*Arthrospira platensis*) using SWE (200 ℃, 20 min, pH 7.0, and 4 MPa) were compared with those isolated using alkaline extraction and aqueous extraction (Du et al., 2020a). SWE showed promising potential in extracting proteins from these materials, particularly pasture grass, where the protein yield and purity were highest for SWE compared to other methods (Du et al., 2020a). Saravana et al. (2016) documented the highest protein recovery, with a maximum total amino acid content of 762 mg/g, from brown seaweed (*Saccharina japonica*) when optimum SWE conditions (180 °C and 1.3 MPa) were employed for extraction. The extracted protein was rich in essential (valine and lysine) and nonessential (glycine, aspartic acid, tyrosine, and glutamic acid) amino acids (Saravana et al., 2016). In another study, Park et al. (2019) demonstrated the preparation of a protein extract from laver (*Pyropia yezoensis*) using SWE at varying conditions (120 to 230 °C and 3 MPa). The authors reported an increase in protein content with elevated temperature; however, free amino acid content was reduced when the temperature exceeded 120 °C. A higher protein content was found in the aqueous phase than in the solid phase when microalgae (*Chlorella pyrenoidosa*) were subjected to SWE, suggesting that protein was solubilized from the food matrix into the aqueous environment. However, the efficacy of SWE depends on temperature, extraction time, and microalgal biomass loading (Zainan et al., 2020).

Furthermore, Sunphorka et al. (2012) observed that temperature was a significant factor influencing protein extraction yield in de-oiled rice bran subjected to SWE, with the parameters of 4 MPa, 250 °C, and 60 min being selected as the optimized conditions. Awaluddin et al. (2016) investigated the use of SWE in extracting proteins from microalgal *Chorella vulgaris.* The results

showed that a maximum total protein yield of 31.2 g/100 g was achieved under the process conditions of 277 °C, 4 MPa, 5% microalgal biomass loading, and 5 min extraction time. According to the SEM analysis, protein extracted from the SWE untreated sample appeared intact and agglomerated, forming a large spherical cell mass. However, SWE-treated samples appeared to be ruptured and segregated into individual particulate cells. This could be due to the non-polar compounds in *C. soyvulgaris* biomass being dissolved in water, with an increase in water temperature resulting in the segregation and disruption of biomass cells.

Functional properties and structural changes are crucial for SWE-produced proteins to be applied in new food formulations. Zhang et al. (2015b) reported that protein isolated from soybean using SWE, performed under a temperature ramp from 120 °C to 225 °C for 15 min, exhibited greater solubility at 120 °C and 200 °C compared with untreated soybean protein isolates. Additionally, the emulsifying index, foaming ability, and foam stability were also higher with increasing SWE temperature. This could be associated with protein unfolding and subsequent exposure of hydrophobic groups mediated by SWE, which was evident in the collapsed crystalline structure of the soybean protein isolates (Zhang et al., 2015b). Similar findings were reported for zein protein isolation by Zhang et al. (2018a), who documented that SWE carried out at temperatures and times ranging from 110 to 170 °C and 20 to 120 min, respectively, was an effective method to improve the functional properties, including foaming capacity, foam stability, and solubility. In addition, SWE improved the mean particle size, thermal stability, and denaturation temperature of zein protein, and increased the ordered structural elements within the protein molecule (Zhang et al., 2018a). Furthermore, the antioxidant, foaming, and emulsifying properties of protein hydrolysate produced from red seaweed (*Kappaphycus alvarezii*) using SWE with different solvents (water, 1% sodium hydroxide, and 1% formic acid) and at varying

conditions (150 to 300 °C/10 MPa) were investigated by Gereniu et al. (2017). The authors reported that the extract exhibited increasing antioxidant properties with increasing temperature and pressure, regardless of the extraction medium used. Although SWE extracted over 80% protein from the starting materials, its content was not influenced by the medium or processing parameters (Gereniu et al., 2017). Pangestuti et al. (2019) documented that the antioxidant properties of protein extract produced from red seaweed (*Hypnea musciformis*) using SWE were dependent on the solid-to-liquid ratios and temperature used. The increased antioxidant properties were attributed to the precipitation of reversibly bonded phenolic compounds with proteins during SWE.

Plant protein extraction using SWE, combined with other environmentally friendly extraction methods, such as enzymatic processes, has also been demonstrated. Lu et al. (2016) investigated the efficacy of protease M hydrolysis (concentration at 4% w/w, pH 4.5, temperature of 50 °C for 10, 30, 60, 90, and 120 min) followed by SWE for extracting protein from heat-denatured soybean meal. A significant increase (59.3%) in extraction yield was observed with SWE treatment (120 °C for 20 min) compared to alkaline-acid precipitation (16.40%) at pH 9.0 and a temperature of 25 °C. However, no changes were observed in the purity of the extracted proteins, which remained at 80%. Nonetheless, the protein extracted using combined treatments had a remarkable improvement in emulsifying ability when compared to its untreated counterpart (Lu et al., 2016). Therefore, SWE is an effective and environmentally friendly technology that can be used individually or in combination with other extraction methods to enhance the extraction yield and techno-functional properties of the isolated protein. However, SWE has its own limitations; the high cost of implementation is a major drawback, particularly during industrialization.

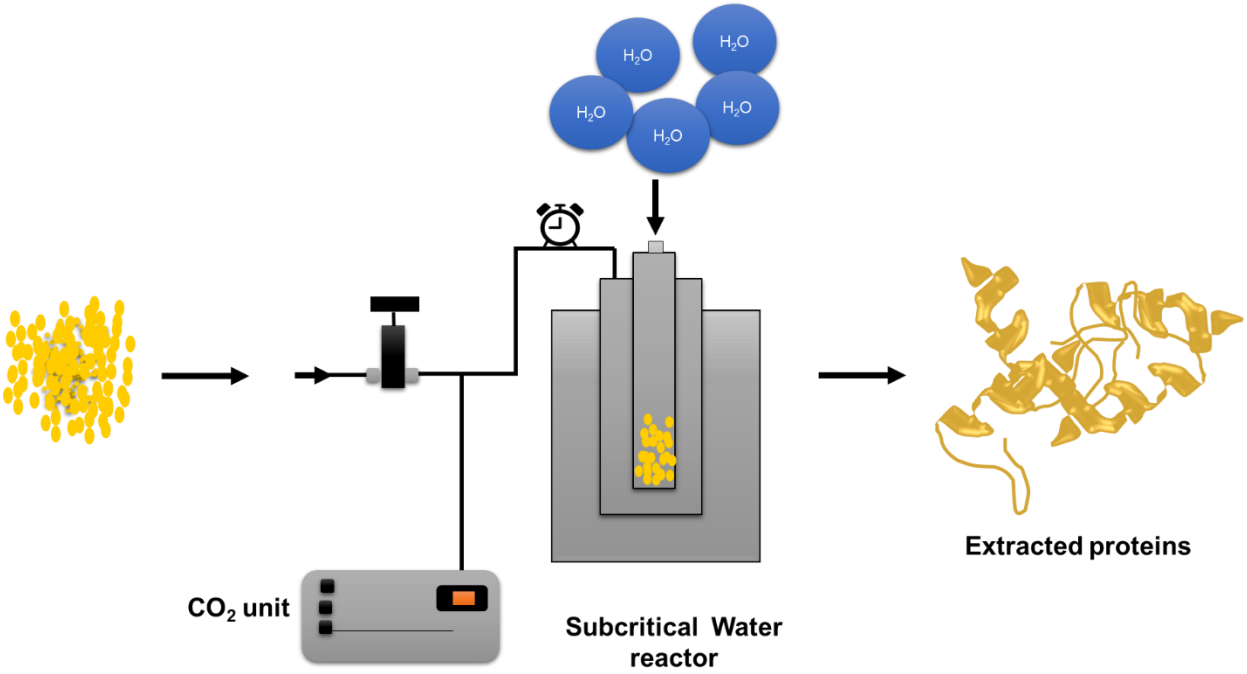

Figure 2. 6. Schematic diagram of Subcritical water extraction (SWE) protein extraction's principle.

2.5.7. High-pressure-assisted extraction (HPAE)

High-pressure processing (HPP) has shown processing potential not only as an innovative technology for food preservation (Olatunde & Benjakul, 2018) but also for food processing (Khan et al., 2019). This technique has existed since the early 1900s and was designated by a French Scientist, Blaise Pascal, who worked on high-pressure processing fluids in the seventeenth century (George & Rastogi, 2017). Compared to classical solvent extraction, high-pressure processing-assisted extraction offers several advantages, including minimal energy requirement, uniform treatment, no toxicity, minimal heat generation, and low thermal degradation of protein structure and activities (Khan et al., 2019; Olatunde & Benjakul, 2018). Furthermore, microorganisms are inactivated during HPP, which can improve the shelf stability of extracted products. During the commercial processing of food by HPP, solid or liquid foods are subjected to a range of pressures,

usually between 100 and 1000 MPa, for a certain period, typically from a millisecond pulse to above 20 minutes. HPP utilizes the “isostatic principle,” which involves applying pressure evenly and instantly to a material from all directions, regardless of its volume and composition. When the pressure is released, the volume of the material reverts to its initial dimension (Daher et al., 2017). During this process, covalent and non-covalent bonds are broken, which might trigger structural changes in the materials. Protein extraction conditions make cells more permeable, facilitating the mass transfer from plant tissue (Khan et al., 2019). Several factors, including pressure, temperature, type and amount of solvent, number of cycles, and extraction time, influence the effectiveness of extraction and recoveries of HPAE (Chen et al., 2009).

The efficacy of HPAE on protein extraction yield has been investigated. Altuner et al. (2012) employed high-pressure processing (220 and 330 MPa for 10 and 30 min at room temperature) to extract pollen protein and compared its protein recovery rate to that obtained using the conventional extraction method (salt precipitation). The authors reported a more than 20-fold increase in protein yield for HPP treatment compared to conventional extraction methods. This was attributed to the deformation of the air bladders' surface when pollen was subjected to HPP treatment. The pollen wall deformation enhanced the leaching of protein into the extraction media. Additionally, protein recovery from pollen was found to depend on both pressure intensity and exposure time (Altuner et al., 2012). Similarly, a higher protein yield was recorded in an intensity-dependent manner when high-pressure homogenization (HPH) at different intensities (50, 100, and 150 MPa) was used for protein extraction from soybean okara, compared to the yield without HPH treatment (Fayaz et al., 2019). Furthermore, a 10.3-fold higher protein recovery yield was recorded when HPH (150 MPa at a different number of passes nP = 1–10) was used for protein extraction from microalgae compared to water extraction. The increased protein yield was associated with

the undifferentiated release of intracellular contents from microalgae cells subjected to scalable cell disruption induced by HPH (Carullo et al., 2018). In addition, O'Connor et al. (2020) demonstrated the efficacy of HPP for protein extraction from different species of macroalgae namely *Fucus vesiculosus*, *Palmaria palmata, Alaria esculenta*, and *Chondrus crispus*. The authors reported that the capacity of HPP in disintegrating cell membranes makes it a promising technology for extracting protein from seaweeds.

The combination of HPAE with other technologies has been implemented to increase the efficacy of protein extraction further. Dong et al. (2011a) investigated the efficacy of high-pressure homogenization under atmospheric pressure (0.1 MPa) and high pressure (40 or 80 MPa), as well as enzymatic treatment using Alcalase on protein recovery from peanuts. The authors reported an increasing protein yield with increasing pressure, attributed to the augmented contact surface area of protein molecules exposed to the extraction mediated by high-pressure processing treatment. Furthermore, Plazzotta et al. (2021) evaluated the efficacy of HPH in combination with the alkaline-acid recovery method for extracting protein-enriched soy okara powders. The authors reported that the protein yield obtained when HPH treatment was almost twice that obtained without HPH treatment. However, the protein recovery was dependent on the intensities used. This was attributed to the fibrous matrix disruption mediated by the intense mechanical forces produced by HPH, which favor the release of tightly packed cell components (Plazzotta et al., 2021).

The impact of high-pressure processing on the techno-functional properties of plant-based proteins has also been documented. The techno-functional properties, including solubility, foaming, gelling, and emulsifying properties of soy okara protein extracted with HPH, regardless of the intensity used, were higher than those prepared without HPH (Plazzotta et al., 2021). In a recent study by Luo et al. (2022), the impact of HPH at different intensities (10, 30, and 50 MPa)

on the techno-functional properties of quinoa protein isolate (QPI) was investigated. The solubility of QPI was found to increase with increasing pressure intensity, which was attributed to the reduced protein particle size after HPH treatment. The interfacial properties (foaming and emulsifying properties) of the HPH-treated QPI were greater than those of the untreated QPI. This was associated with the unfolding and/or partial dissociation of protein structures mediated by HPH treatment, as confirmed by increased surface hydrophobicity and free SH groups. However, HPAE has demonstrated great potential in extracting plant-based proteins. The scale-up of this technology is still in the infancy stage. The extraction efficacy of HPAE depends on pressure intensities, time, and many other factors. However, when harsh conditions are used, extracted protein may often undergo conformational changes and protein oxidation. These structural changes may negatively influence the techno-functional properties of the extracted protein. Therefore, optimizing the processing parameters for each plant material using this technology is imperative.

2.5.8. Pulsed electric field-assisted extraction

Pulsed electric field (PEF) is an environmentally friendly technology that has garnered considerable attention in the food industry in recent years. PEF has shown promising potential in many applications, such as food preservation, pesticide residue degradation, stimulating active components in biosynthesis, and biomolecule extraction (Olatunde & Benjakul, 2018; Zhang et al., 2021b). Furthermore, when compared to conventional solvent extraction techniques, PEF has a greater average due to its nonthermal property, less processing time, chemical-free processing, lower energy consumption, improved yield, and environmental friendliness (Ranjha et al., 2021), which overall promoted this technology as a novel technology for plant-based protein extraction.

PEF technology utilizes electric waves with high voltage amplitudes (typically 10–80 kV/cm), in short, electrical impulses (from microseconds to milliseconds each), for the treatment

of the product placed between the electrodes in the chamber (Olatunde & Benjakul, 2018). Depending on the voltage used, PEF can cause electroporation (100-300 V/cm, batch mode) and electro-permeabilization (20-80 kV/cm, continuous mode) (Ranjha et al., 2021). In the former, high-voltage electric field pulses are applied to the targeted material, causing the breakdown of the cell membrane and facilitating protein extraction through increased mass transfer from the raw material, mediated by the opened channels (Xi et al., 2021). However, electro-permeabilization promotes chemical reactions from solvents and ingredients to enhance their solubility. Simply put, electro-permeabilization is caused by the high and swing resonance generated from the high electric field (Sánchez-Vega et al., 2015; Xi et al., 2021). Nevertheless, the efficacy of PEF depends on the process conditions such as electric field, strength (kV/cm), pulse frequency, pulse width, the shape of the pulse wave, and exposure time (related to the flow rate and volume of fluid in the electrode chamber) (Olatunde & Benjakul, 2018).

The application of PEF for extracting plant-based proteins has been demonstrated. Yu et al. (2015) employed PEF with moderate (800 V/cm for 200 ms) and high electrical field intensities (5 kV/cm and 20 kV/cm for 2 ms) at room temperature (20 °C) to treat rapeseed stems and leaves. The authors concluded that the electric field intensity of 20 kV/cm could improve the protein yield from rapeseed leaves up to 80%. It was suggested that a threshold of electrical field strength must be exceeded to achieve a high protein yield. Furthermore, it was stated that PEF technology could also enhance the nutritional value of rapeseed stems and leaves in the animal feed industry (Yu et al., 2015). Similarly, Jaeschke et al. (2019) used specific treatment energies (28, 56, and 112 J/mL) at room temperature (25 °C) for the treatment of *Arthrospira platensis* prior to protein extraction. It was observed that the protein yield increased significantly from 74% to 105% with increasing energy intensity. This was attributed to the increased electroporation process, which occurred with

increasing energy intensity, promoting cell membrane disruption and resulting in high protein recovery. Furthermore, the electroporation process can promote the selective extraction of water-soluble compounds, which is considered a primary advantage of PEF (Jaeschke et al., 2019). In addition, Polikovsky et al. (2016) demonstrated the extraction of protein from the marine macroalgae Ulva using PEF treatment. In this study, the application of an electric field strength of 2.964 kV/cm and a pulse duration of 5.70 μs resulted in a nearly three times higher protein concentration in the extracted juice compared to the untreated counterpart.

Its combined treatment with other technologies has been investigated to further enhance the protein extraction efficacy of PEF. Polikovsky et al. (2019) documented the protein extraction from green microalgae *Ulva spp* by osmotic shock combined with PEF and hydraulic pressure. The authors found that the highest protein yield, 53.8 μg/mL, was obtained when PEF with 26 kV, 7.26 kV/cm, 50 pulses, and 2.3 μs was used. Moreover, Prabhu et al. (2019) coupled the PEF treatment with the mechanical press technique on marine macroalgae *Ulva ohnoi* and found 4 times higher protein content in the PEF (1 kV/cm, 3 Hz, 200 pulses, 50 μs) treated sample (329.62 mg/L) compared to the pressed alone (84.58 mg/L). A similar observation was documented when the same combined technologies were used for protein extraction from blackberries (Barba et al., 2015).

The effect of PEF on the techno-functional properties of plant-based protein has also been elucidated. Li et al. (2007) reported that the solubility of soy protein isolates (SPI) was augmented with increasing PEF strength up to 30 kV/cm and a treatment time of 288 μs. It was postulated that the increase in PEF strength and time facilitated the partial unfolding of SPI, exposing the previously hidden hydrophobic groups, which led to increased interactions between protein molecules and water. In the recent study by Zhang et al. (2017a), the effect of PEF treatment on

the functional properties of canola protein was investigated. It was observed that solubility, water-holding capacity, emulsifiability, emulsion stability, oil-holding capacity, foamability, and foam stability were increased upon the application of PEF treatment compared to the untreated group. This was attributed to the exposure of inner hydrophilic or hydrophobic groups through the unfolding of protein molecules induced by PEF. Based on the available literature, PEF can induce protein structural modifications, which may improve the techno-functional properties of plant-based proteins. However, there is insufficient information on the mechanism of action of PEF on protein functionality. Therefore, further research is needed to fully elucidate the PEF mechanism related to protein functionality from various plant sources. Although PEF is a promising green processing technology, it still faces scaling-up challenges. There is limited fundamental research on optimized process conditions for a variety of cash crops. Also, because PEF is compatible with easily flowing material (liquid materials), it is required that a special treatment chamber must be constructed for PEF to accommodate solid materials, which adds to the high cost of setting the PEF processing system. This would be the major obstacle for the industrial application of this method.

2.6. Current challenges and future perspectives

New eco-friendly extraction technologies offer advantages in terms of improved safety, enhanced extraction yield, and reduced negative impact on the techno-functional properties of food proteins compared to conventional methods. However, most of these methods have only been tested on a laboratory scale, so commercial adaptation is still in its early stages. Therefore, it is imperative to conduct substantial research and development activities to understand, optimize, and apply these complex processes, thereby increasing their application in the food industry, particularly in the plant protein sector. Therefore, it is crucial to assess the economic viability and

cost-effectiveness of scaling up these new, environmentally friendly technologies. Furthermore, the possible mechanisms or modifications of protein structures must be studied using the new extraction methods, as they dramatically affect protein physicochemical and functional properties. In addition, particular attention must be placed on energy efficiency, environmental impact, and co-product generation in order to develop new technologies with enhanced process efficiency, product quality, and environmental sustainability.

Table 2.1. Summary of extraction yield and functional properties of novel extraction technologies over the conventional methods

Novel protein extraction technique	Plant materials	Extraction optimum conditions	Protein content recovered (PC)/ Yield (Y) from the conventional method	Protein content recovered (PC)/ Yield (Y) with novel extraction method	Improved Techno-functional properties	Reference
1. Enzyme -assisted Extraction	Soybean meal (SBM, rapeseed meal (RSM), microalgae meal (MAM)	Endoprotease enzyme (Protex 40XL, Protex P, Protex 5L and Protex 26L, Protex 50FE), Incubation- 24 hrs at 40 ℃, pH-12.0, protein concentration- 5%	SBM- Y- 80% RSM and MAM- Y- 15-30%	SBM- Y- 90%, RSM-MAM- Y- 50-80%		Sari et al. (2013)
	Defatted soybean flakes	β-glucosidase, phytase-pH- 4.5, T- 50 ℃, Time-30 min (Alkaline extraction- pH-8.0, 2 hrs)	Y-65.18% PC-87.99	Y-66.60%, PC-81.06%		Wei et al. (2018)
		β-glucosidase, phytase, and acid protease- cocktail enzyme-assisted countercurrent extraction, pH-4.5, T- 50 ℃, Time-30 min. (Alkaline	Y-65.18%, PC-87.99	Y-74.83%, PC-83.06%	Less flavor volatiles, higher thermal stability	Wei et al. (2018)

	extraction- pH-8.0)				
Defatted soybean grits	Commercial cocktail (cellulase, pectinase, and xylanase), pH 5.5 for 3 hrs at 50 °C solid: liquid ratio 1:10 (w/v)- Enzymatic assisted alkaline extraction	Y- 41.03%	Y-40.87%	Improved solubility, foam capacity, foam stability	Perović et al. (2020)
	Commercial enzyme cocktail (cellulase, xylanase, pectinase, arabinanase, and β-glucanase, pH 5.5 for 3 hrs at 50 ℃, solid: liquid ratio 1:10 (w/v)- enzymatic assisted alkaline extraction	Y- 41.03%	Y-45.93%		Perović et al. (2020)
Flaxseed	Enzymatic solvent extraction, pH-5.0, enzyme concentration- 2%, Temperature – 37 ℃, Time- 4 hrs	PC- 51.05%	PC65.08%	Lower improved functional properties (emulsion capacity, emulsion	Tirgar et al. (2017)

				activity & emulsion stability) compared to alkaline extraction.	
	Enzyme assisted alkaline extraction, pH- 5.0, enzyme concentration- 2%, Temperature – 37 ℃, Time- 4 hrs	PC- 51.05%	PC- 86.8%	High solubility compared to alkaline extraction alone.	Tirgar et al. (2017)
Okara	Viscozyme enzyme (cellulase, xylanase, endogluconase), pH- 6.2, Temperature- 53 ℃, Enzyme concentration- 4%	PC- 47.8% & protein recovery 15.1%	PC- 58% & protein recovery 28%		de Figueiredo et al. (2018)
Red seaweed	Celluclast® 1.5 L, Shearzyme® 500 L, Alcalase® 2.4 L FG, and Viscozyme®, pH- 7.0, enzyme concentration- 0.2, -0.4%, Temperature- 60 ℃ Time- 14 hrs	Protein extraction efficiency- 45%	Celluclast® 0.2% w/w plus Alcalase® 0.2% w/w (90.0% extraction efficiency) Shearzyme® 0.2% w/w plus Alcalase® 0.2% w/w (85.5% extraction efficiency)		Kumar et al. (2021)

	Akebia trifoliata (Thun b.) Koidz. seed	cellulase (0.1 g of 10 U/mg) Temperature- 40 ℃ Time- 2 hrs Ph-10.0	Y- 17.15% PC- 40.89%	Y- 19.38% PC- 52.78%	Improved Water holding capacity and forming and emulsification properties	Jiang et al. (2021)
2. Deep eutectic solvent extraction	Rapeseed cake (RC), Evening primrose cake (EC)	Glycerol: ChCl (2:1), liquid: solid ratio (9:1), 60 - 140 ℃	Y- 41%	Y- 11.5-19.9 g/100g with RC and 8.4-34.2g/100g EC, Protein content of RC-36-48%, the Protein content of EC-40-50%		Grudniewska et al. (2018)
	Brewer's spent grain	90%wt NaAcO: urea (1:2), T-80°C, Time- 4hrs, 10% weight consistency.	PC- 82.70%	Y- 79%		Wahlström et al. (2017)
	Oat protein	ChCl-1,4-butanediol/water (1:3:1), Time - 90min, Temperature- 80°C, Solid: liquid ratio 1:9	PC- 85.4%	PC- 55.28%	Higher thermal stability	Yue et al. (2021)
	Pumpkin seed	PEG 200- based DES concentration 28% w/w, solid to liquid ratio 28 g/mL, microwave power 140 W, and	-	Y- 93.95%	-	Liu et al. (2017)

		extraction temperature 43 °C				
3.Reverse micelles extraction	Soybean flour	Forward extraction: AOT-0.05 M, Soybean flour: reverse micelles phase 1:20 (w/v), Time-30 min, 300 rpm, T- 45°C, pH- 7.5,	Extraction efficiency-28%, PC- 81.63%	PC-85.2%	Improved Nitrogen solubility index, oil absorption capacity, foaming capacity, foaming stability, emulsifying capacity, and emulsifying stability	Zhao et al. (2018)
	Soybean	Forward extraction: AOT (12g in isooctane 200mL) extraction time of 25 min, pH of 3.0, the temperature of 45 °C, KCl concentration of 10.0 mol/ L	-	Extraction effcieny-48.66%	Improved emulsion activity index and stability index	Zhang et al. (2021a)
		Backward extraction: AOT (12 g in isooctane 200 mL) extraction time of 10 min, pH of 4.0,	-	-	Improved emulsion activity index and stability index	

	the temperature of 30 °C, KCl concentration of 0.5 mol /L				
Soybean	AOT concentration- 0.06 g/mL, KCL concentration- 0.05 g/mL, pH- 8.5, time- 30 min, temperature- 40 °C	11S globulin- PC- 83.75%	11S globulin PC- 87.56%	Lower surface hydrophobicity and high storage and loss modulus in gel formation than the conventional method,	Yao et al. (2021)
Peanut	Backward extraction: AOT concentration- 80 g/L, pH-7.5, time- 20 min, temperature- 35 °C, ion strength- 1.1 mol/L	-	extraction efficiency- 79.03%	-	Guo et al. (2015)
Jackfruit flour	Temperature- 25 °C, stirring time of 120 min, the mass of flour of 100 mg, and a ratio H2O SDS- 1 of 50	-	extraction efficiency- 79.00%	-	Reis et al. (2016)

Walnut	Microwave assisted Backward extraction: AOT concentration- 0.08 g/mL, pH- 7.5, time- 30 min, temperature- 45 °C	-	Extraction efficiency- 95.43%	Improved solubility, water, and oil holding capacity, emulsion, and forming properties.	Wang et al. (2021)
Sesame seed	Forward extraction with AOT. Isooctane, KCl, and backward extraction	Extraction efficiency 24-37%	PC- 86.2%	Improved Solubility, emulsifying capacity, and emulsion stability	Zhao et al. (2012)
Defatted wheat germ (DWG)	Backward extraction: Temperature > 25 °C, Time-60 min, pH-8.0, 400 rpm, Vacuum drying- 30 °C		Extraction efficiency- 30%, PC- 98.38%	higher nitrogen solubility index, fat absorption capacity, foaming capacity, foaming stability, and emulsifying stability	Zhu et al. (2010)
Defatted wheat germ (DWG)	Forward extraction: AOT conc entration- 0.06 g/mL, KCl concentration- 0.1 mol/L, Time- 30	Y- 2.92%	Extraction efficiency- 37%		Sun et al. (2008)

		min, T- 36 °C, pH- 8.0, W0 25				
	Grape seed protein	Forward extraction: Ceryl-trimethyl-ammonium bromide -39 mmol/L, NaCl concentration- 0.01 mol/L, Crude protein concentration- 2.1 mg/mL, pH-5.6.	PC-74.10%, Y-11.0%	Extraction yield-82.3%		Zhang et al. (2017b)
4. Microwave-assisted extraction	Rice bran	MW power- 1000 W, Time- 90 sec, Rice bran: water (0.89 g/10mL)		C- 77.27%, Protein recovery- 22.07%, Y-4.37%	Water & oil absorption capacity,	Phongthai et al. (2016)
	Defatted rice bran (DRB)	MW power- 350-400 W, Time-90 s, T- 40 °C, DRB: water (0.5:10), particle size-175µm		PC- 75.9%, Y- 12.2%	No significant changes in nitrogen solubility index or digestibility compared to conventional methods	Bedin et al. (2020)
	Peanuts flour (PNF)	MW power- 725 W, Time- 8 min, PNF: water (1:10)		Y- 55.53%, PC- 100%	Water absorption Index, foam activity, emulsifying activity, and	Ochoa-Rivas et al. (2017)

				in vitro digestibility	
Soybean Milk	MW power-675 W, Temperature-80 °C, Stirring-160 rpm	Y- 3.86 kg soybean milk/kg of soybean, PC- 13.11%	Y- 4.86 kg soybean milk/kg of soybean, PC-7.38%	Improved viscosity, whiteness index, protein solubility, and protein digestibility	Varghese and Pare (2019)
Microalgae Chlorella vulgaris	30% ammonium sulphate, 1:1 ratio of slurry to t-butanol, 120 sec microwave time, 80% microwave duty cycle, 100 W microwave power, and 0.5 % (w/w) microalgae biomass concentration	Y (Three phase partitioning (TPP) system) – 24.9%	Y- (Microwave wave TPP)- 63.2%	-	Chew et al. (2019)
Jackiopsis ornata roots	26:1 (v:w) liquid to solid ratio, 0.0284 radius particle size, 65 °C temperature, 300 W microwave power, and 20 min extraction time		Y- 20.47%	-	Elhag et al. (2019)
Watermelon seed	50 W microwave power, 1:30 solid to solvent ratio,	Protein recovery rate- 72%	Protein recovery- 90%	Similar water holding capacity	Behere et al. (2021)

		pH-10.0, and Time- 2 min			decreased oil holding capacity and improved forming properties	
	Coffee silverskin	Microwave power of 434.7 W, time – 10 min, solvent concentration-0.6 M	Protein recovery- 24.35%	Protein recovery- 43.53%	-	Wen et al. (2021)
	Cold-pressed sesame seed meal	Temperature-87.8 ºC, pH 8.0, and 37 min time, Microwave power- 850 W	-	-	Improved oil and water holding capacity and forming properties and protein digestibility than the raw sample	Sá et al. (2022)
Ultrasound assisted extraction	Rape seed meal	Time- 84 min, temperature- 35 ℃, Power- 450 W, solid: liquid ratio- 1:24, pH-11.5		Extraction efficiency- 76.83% (35.43% increase compared to water bath extraction), PC increased by 8.31%	Improved oil adsorption ability, emulsifying capacity, foaming capacity, and foam stability	Dong et al. (2011b)
	Ganxet beans (Phaseolus vulgaris L. var. Ganxet)	US power- 40 kHz, 250 W, Time- 60 min, Temperature-4	Y- 24.02%, Protein recovery- 50.17%	Protein recovery- 78.73%, Y- 37.98%	Improved Water holding, oil holding	Lafarga et al. (2018)

	℃, pH- 12.95, solid: liquid- 1:10			capacity, foam capacity, and emulsificatio n capacity were comparable to those of other vegetable-derived proteins	
Sunflower meal	Power density- 220 W/L, Temperature- 45 ℃, Time-15 min, Sample: solvent ratio- 0.5/10 (w/v), pH- 8.0		Y-54.26%, PC-934.92g/kg	Improved Oil holding capacity, protein solubility, emulsificatio n activity, and stability	Dabbour et al. (2018)
Coconut milk	Time-2.5 min; Frequency- 24 kHz; Power- 0.573 kW/kg,	-	Y- 49.6–86.1%	-	Martínez-Padilla et al. (2022)
Watermelon seed	pH-9.0, powder to solvent ratio -1:50 w/v, temperature - 30 ℃, ultrasound power- 90 W, frequency -25 kHz, and 75% - duty cycle	Y-75%	Y-87%	Improved Water and oil holding capacity, emulsificatio n properties, and protein digestibility	Gadalkar and Rathod (2020)

	Defatted perilla seed meal	Extraction temperature of 40 °C, power of 61 W, extraction time of 12 min, and the ratio of liquid to solid of 40 mL/g	-	Y-10.77%	-	Zhu and Fu (2012)
	Blue-green microalgae Spirulina platensis	Temperature- 45 °C, pH- 7.46, and time- 120 min.	-	Y-29.05%	Improved protein digestibility	YuCetepe et al. (2018)
	Pomegranate seed	Extraction time- 5 min and amplitude- 20%,	Y-15.3%	Y-10.2%	Improved protein solubility	Guzmán-Lorite et al. (2022)
	Cold-pressed sesame seed meal	US frequency- 40 kHz, power density- 135 W/l, Temperature-87.8 ºC, pH 8.0, and 37 min time	-	-	Improved oil and water holding capacity and forming properties and protein digestibility than the raw sample	Sá et al. (2022)
Subcritical water extraction	Watermelon	Sample: solvent ratio- 1:50 (w/v), Temperature-30 °C, Time-9 min, power- 90 W, frequency- 25 KHz, pH- 9.0, duty cycle- 75%	Y-75%	Y- 87%	Improved Water holding, oil holding capacity, and protein digestibility	Gadalkar and Rathod (2020)

Soybean isolates	Solid : water – 1:200, Time- 20 min, T- 200 ℃	-	-	Improved Solubility, emulsifying, and foaming properties	Zhang et al. (2015b)
Zein protein	Solid : water – 1:60, Time- 20-120 min, T- 110-170 ℃	-	-	Improved Solubility, foam capacity, and foam stability	Zhang et al. (2018a)
Ground oilseed cakes of hemp, pumpkin, and flax seed	The sample-to-solvent ratio of 1:30, Pressurization- 99.999% N2 or CO2 to 2.0 MPa at 160 °C for 1 hrs.	-	Pumkin (N2+0.05mol/L HCl) protein yield– 60.17% Hemp (N2+0.05mol/L HCl) protein yield - 40.45% Flax seed (N2+0.05mol/L HCl) protein yield -53.02%	-	Švarc-Gajić et al. (2020)
Brewer's spent grain (BSG), pasture grass (PG), and cyanobacteria (Arthrospira platensis; AP)	Extraction time- 20 min, Temperature- 200 °C, pH- 7.0	Protein yield BSG- 21.4% PG- 1.1% AP- 55.5% Protein content BSG- 60.2% PG- 9.8% AP- 68.8%	Protein yield BSG- 7.2% PG- 6.7% AP- 19.9% Protein content BSG- 36.7% PG- 15.7% AP- 58%	Lowest interfacial tension by AP extracted by subcritical water	Du et al. (2020a)
Brown seaweed; Saccharina japonica	The temperature of 180 -420 °C and pressure between 1.3- and 52 MPa	-	Y- 72.21% to 98.91%	The decreasing	Saravana et al. (2016)

					trend of viscosity	
	Red algae; Pyropia yezoensis	1:20 solid to liquid ratio, (120 to 230) °C temperature, pressure 30 MPa	Hot water extraction (protein content)- 9.39 g BSA/100g dried mass Ethanol extraction-(protein content)- 6.96 g BSA/100g dried mass	Y- 58.89 % to 78.13%	-	Park et al. (2019)
	Microalge; Chlorella pyrenoidosa bio mass	Temperature-270 ℃, 10 min extraction time, and 1% of microalgae loading	-	Y- 18.773%	-	Zainan et al. (2020)
	De-oiled rice bran	7 MPa pressure, Temperature-250 °C, and time- 60 min	-	Extraction efficiency- 100%	-	Sunphorka et al. (2012)
	Chlorella vulgaris	Temperature-277 ℃, 5% of microalgal biomass loading, and 5 min extraction time	-	Y- 31.2%	-	Awaluddin et al. (2016)
High pressure assisted-extraction	Cedrus atlantica pollens	330 MPa for 30 min	The total amount of proteins was 1.946 µ g/mL	The total amount of protein- 25.9270 µ g/mL	-	Altuner et al. (2012)
	Soybean okara	Okara dispersions (10 g/100 g), 150 MPa, 5 passes	-	Y- 90%	Higher viscosity	Fayaz et al. (2019)

Chlorella Vulgaris	Suspensions (1.2%, w/w), 150 MPa, 5 passes	-	54.1% (w/w) of total proteins	-	Carullo et al. (2018)
Brown macro algae; Fucus vesiculosus	600 MPa of pressure for 4 min, 20 g/L water algae dispersions	Y- 35.1%	Y-23.7%	-	O'Connor et al. (2020)
Brown macro algae; Alaria esculenta	600 MPa of pressure for 4 min, 20 g/L water algae dispersions	Y- 18.2%	Y-15%	-	O'Connor et al. (2020)
Red macro algae; Palmaria palmata	600 MPa of pressure for 4 min, 20 g/L water algae dispersions	Y-12.5%	Y-14.9%	-	O'Connor et al. (2020)
Red macro algae Chondrus crispus	600 MPa of pressure for 4 min, 20 g/L water algae dispersions	Y-35.2%	Y-16.1%	-	O'Connor et al. (2020)
Soy okara powders	50−100 MPa pressure, 5 passes, Okara dispersions 20 g/100 g of water	PC- 80%	PC-68-66%	Good balance of solubility, foaming, gelling and emulsifying properties	Plazzotta et al. (2021)
Quinoa protein isolates (QPI)	5% w/w QPI suspension, Temperature- 33 °C, 5 passes, pH-	-	-	Improved emulsifying and forming capacity,	Luo et al. (2022)

		7.0, 0-50 MPa pressure			enhanced the solubility and viscoelasticity	
Pulse electric field assisted extraction	Rapeseed leaves	Electric field strength E = 20 k V/cm, time -2 min, temperature-20 °C	-	Y- up to 80%	-	Yu et al. (2015)
	Arthrospira platensis	56 and 112 J /mL energy level, temperature (25 °C). 6 hrs incubation period	-	Y-74-105%	-	Jaeschke et al. (2019)
	Macroalgae Ulva	75 pulses with an average electric field strength of 2.964 kV/cm, and pulse duration of 5.70 µs	Protein concentration-23.80 µg/ mL	Protein concentration-59.13 µg/ mL	-	Polikovsky et al. (2016)
	Soybean protein isolates	PEF strength 30 kV/cm, treatment time 288 µs	-	-	Higher solubility, surface SHF, and hydrophobicity	Li et al. (2007)
	Canola seed protein	35.00 kV of voltage, 185 sec of residence time, 806 Hz of pulse frequency, and 8 µs of the pulse width.	-	-	Improved solubility, water-holding capacity, emulsibility, emulsion	Zhang et al. (2017a)

				stability, oil-holding capacity, foamability, and foam stability

Chapter 3

Improved protein extraction technology using deep eutectic solvent system for producing high-purity fava bean protein isolates at mild conditions

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3.1. Abstract

This study aimed to extract fava bean proteins using an eco-friendly deep eutectic solvent (DES) system composed of choline chloride and glycerol. Protein extraction conditions were optimized using response surface methodology, and the extracted protein was characterized in comparison to that extracted using a conventional alkaline solution. The optimum conditions obtained using the RSM model were: 28:1 w/w liquid: solid ratio, 1:2 w/w choline chloride: glycerol molar ratio, 50 ºC temperature, 1 hrs extraction time, and 40% w/w water content. Under these optimum conditions, protein content, protein yield, and protein recovery rates of 92.33 ± 2.28%, 65.42 ± 6.53%, and 23.15 ± 2.31%, respectively, were achieved. Alkaline extraction showed similar protein content (92.50 ± 1.36%) but lower protein yield (60.76 ± 1.16%) and recovery rate (21.74 ± 0.19%) when compared to DES-extracted proteins ($P < 0.05$). The secondary structure analysis revealed an increased α-helix (21.37%) content in DES-extracted proteins compared to alkaline-extracted proteins (10.68%). Moreover, the alkaline-extracted proteins exhibited intermolecular β-sheets as protein aggregates (7.61%), along with an increased percentage of β-turns (19.71%). The molecular weight distribution pattern of proteins extracted from both methods showed larger similarities. Overall, DES protein extraction could serve as an alternative to the conventional alkaline extraction method, thereby promoting sustainable plant protein production from fava beans for use in novel food formulations.

3.2. Introduction

The global demand for protein ingredients is increasing steadily at a rapid pace. In 2020, the global protein ingredient market was worth US$ 38.5 billion, and it is expected to grow at 10.5% from 2021 to 2028 (Grandview Research, 2021). This has increased the need for alternative protein ingredients in the food industry. Fava bean (*Vicia faba* L.) is a legume crop belonging to the *Fabaceae* family, rich in protein (26-35%) (Sofía et al., 2021). However, fava bean consumption as human food is limited, especially in developed countries, as they heavily rely on meat as a primary protein source (Multari et al., 2015). In Canada, the fava bean is mainly grown for animal feed and is less consumed in the human diet; therefore, it is considered an underutilized crop. However, fava bean has excellent potential to be used as a sustainable and alternative protein source that could be fully exploited in novel food applications to increase value-added utilization.

Conventional alkaline extraction is the most commonly employed method in the food industry for producing protein ingredients from plant sources and is well-documented in the scientific literature (Hewage et al., 2022; Nadeeshani et al., 2022). However, conventional protein extraction methods are often associated with numerous limitations and environmental concerns. For example, alkaline extraction generates substantial amounts of wastewater, which has a negative impact on the environment. Also, high consumption of chemicals and water increases production costs (Sun & Bandara, 2019). On the other hand, the extreme pH, ionic strengths, and temperatures used during alkaline extraction lead to undesirable changes in the techno-functional properties of proteins due to protein denaturation (Sofía et al., 2021; Sun & Bandara, 2019). Therefore, the search for alternative green technologies to replace the extensive use of conventional, environmentally hazardous solvents appears to interest researchers. In this context, deep eutectic solvents (DES) have recently drawn attention as a new type of green extractant in

the scientific community due to their less toxicity, eco-friendliness, biodegradability, and low cost (Zainal-Abidin et al., 2017). The application of DES has been expanded in a wide range of fields such as catalysis (Chen et al., 2023; Williamson et al., 2017), biomass pre-treatment (Chen & Mu, 2019; del Mar Contreras-Gámez et al., 2023), organic synthesis (H. Sun et al., 2020; Thakur et al., 2022), drug delivery (Li et al., 2023; Pradeepkumar et al., 2019) and extraction of biomolecules (Yue et al., 2022; Yue et al., 2020). In biomolecule extraction, DES has gained increased attention for extracting various bioactive molecules, such as polyphenols, phenolic acids, and flavonoids (Zainal-Abidin et al., 2017). Recently, the application of DES in plant protein extraction is gaining growing interest as a promising sustainable solvent for protein extraction.

Grudniewska et al. (2018) reported the extraction of protein from rapeseed and evening primrose cake using a glycerol (Gly) and choline chloride (ChCl) DES-based system (1:2 molar ratio). They achieved 36-48% and 11.5-19.9% protein content and protein yield, respectively, for rapeseed cake and 40-50% and 8.4-34.2% for evening primrose cake. In another study using oats, a 1:3:1 ratio of ChCl-butanediol and its water binary mixture yielded a 55.28% protein content and a 7.98% extraction yield when heated to 80 ºC for 90 min, with a solid: liquid ratio of 1:9 (Yue et al., 2021). Moreover, Wahlström et al. (2017) demonstrated improvements in protein yield (79%) and protein content (54.7%) for Brewer's spent grain using a 90 wt% (weight basis) NaAcO and urea DES system. In addition, Lin et al. (2020) demonstrated a significantly higher protein yield (39.16 ± 1.22 mg/g) for bamboo shoots using ChCl and levulinic acid DES system, compared to alkaline extraction (23.88 ± 1.10 mg/g). Based on these reported studies, the protein content and the extraction yield are comparatively low. However, DES-based protein extraction depends on several factors such as type of raw material, liquid: solid ratio, type of DES system and its physiochemical properties, molar ratio, extraction temperature, pH, and time (Liu et al., 2018;

Vilková et al., 2020). In addition to these factors, the addition of water to the DES system also plays a crucial role. Water can alter the polarity of the DES system by enhancing the extraction process through an increased rate of dissolving target compounds (Vilková et al., 2020). Therefore, a systematic optimization study is necessary to determine the optimal extraction conditions. So far, ChCl-Gly is one of the first reported DES systems, and it has been well-explored due to its low toxicity, high biodegradability, and low cost (Ferreira et al., 2021). Hence, in our study, we selected a ChCl and Gly-based DES system for the extraction of proteins from fava beans. Thus, we hypothesized that optimizing protein extraction conditions using Gly and ChCl-containing DES system would improve the protein content and extraction yield of fava bean protein. Therefore, the primary objective of this study was to optimize protein extraction conditions using five variable factors: liquid: solid ratio, ChCl: Gly molar ratio, extraction time, extraction temperature, and water content, employing response surface methodology (RSM). The effect of pH on the extraction yield was investigated using a single-factor experiment. The isolated proteins obtained from optimized variable extraction conditions were analyzed for physicochemical characterization and compared to those extracted using the conventional alkaline extraction method.

3.3. Materials and Methods

3.3.1. Materials

Snowbird, non-tannin dehulled fava bean seeds were purchased from Prairie Fava MB, Canada. Choline chloride (> 99.0%), glycerol (> 99.5%), and Bradford protein assay kit were purchased from Fisher Scientific (Ottawa, ON, Canada). The total starch assay kit was purchased from Megazyme Ltd. (Bray, Ireland). Laemmli sample buffer, 2-mercaptoethanol, Precision plus protein standards (10-250 kDa), Coomassie Blue R-250, and precast gels (4-15%) were purchased

from Bio-Rad Laboratories Ltd (Mississauga, ON, Canada). All chemicals used were of analytical grade.

3.3.2. Methods

3.3.2.1. Milling of fava bean seeds

Dehulled fava bean seeds were milled using a Prater-Sterling Impact Mill (M-21, Prater Industries, Bolingbrook, IL, USA) equipped with 0.2 mm screens. The collected flour was stored at -20 °C until further use.

3.3.2.2. Experimental design and statistical Analysis

DES-based fava bean protein extraction was optimized using the RSM statistical model. A half-fraction central composite design (CCD) was used to determine the protein extraction parameters. In the optimization, five independent variables, ChCl: Gly molar ratio (X_1), liquid: solid ratio (X_2), extraction time (X_3), extraction temperature (X_4), and water content (X_5), were evaluated at five levels (Table 3.1) using appropriate ranges based on the reported literature (Lin et al., 2020). The complete design, with five variables, generated 32 runs that included 6 center points (Table 3.2). Protein yield was estimated as a response to evaluate the extraction performance. Each variable was coded according to equation (1).

$$X = x_i - x_0/\Delta_x \quad (1)$$

Where X is the coded value, x_i is the corresponding actual value of the independent variable, x_0 is the real value at the center point, and Δx is the increment of x_i corresponding to X.

The response function was the protein extraction yield (Y, mg/g) and was fitted to a second-order polynomial model, as shown in equation (2).

$$Y = \beta_0 + \sum_{i=1}^{k} \beta_i x_i + \sum_{i=1}^{k} \beta_{ii} x_i^2 + \sum_{i<j}^{k} \beta_{ij} x_i x_j + \varepsilon \quad (2)$$

Where β_0 is the regression coefficient (constant term), β_i, β_{ii}, and β_{ij} are the linear, quadratic, and interaction effects, respectively. Backward elimination of the terms was performed to generate a reduced model where $P < 0.1$ is considered significant. Analysis of variance (ANOVA) was used to evaluate model adequacy, where the coefficient of determination (R^2) and lack of fit were determined. The optimum predicted protein yield obtained by the RSM model was experimentally validated using optimized protein extraction conditions. Statistical Analysis was performed using MINITAB software (MINITAB version 20, LLC, PA, USA). All results were analyzed at a significance level of $P < 0.05$. The significant factorial interaction plot and corresponding contour plots were generated using the function of two factors, holding the other factors constant.

Statistical analysis of extracted protein fractions was tested using one-way ANOVA with Tukey test and t-test to determine significant differences among the mean values. The same software mentioned above was used for statistical analysis with a significance level of $P < 0.05$. All analyses were carried out using three replicates.

Table 3.1. Independent variables at five levels used in the half-fraction CCD design

Input Variables	Levels				
	-2	-1	0	1	2
ChCl: Gly molar ratio (w/w) (X_1)	2	3	4	5	6
Liquid: solid ratio (w/w) (X_2)	10	15	20	25	30
Temperature (ºC) (X_3)	50	60	70	80	90
Time (hrs) (X_4)	1	1.5	2	2.5	3
Water content % (w/w) (X_5)	0	10	20	30	40

Five independent variables used in the central composite design (CCD) in response surface methodology (RSM). ChCl - Choline chloride, Gly- Glycerol

3.3.2.3. Synthesis of DES

DES solvent systems were synthesized by mixing ChCl and Gly at different molar ratios, as indicated in Table 3.1, according to the method described by Grudniewska et al. (2018) with slight modifications. The DES mixtures were heated to 80 ºC and stirred at 500 rpm until a clear solution was obtained. Thereafter, the DES solutions were cooled to room temperature (~22 °C), and the appropriate water content in the synthesized DES was adjusted from 0 to 40% (w/w) (Table 3.1), as specified in the RSM design matrix, as illustrated in Table 3.1.

3.3.2.4. Characterization of physicochemical properties of DES

The pure DES (100% DES w/w) and the optimized DES (40% water w/w and 60% DES w/w) with a molar ratio of 1:2 (choline chloride: glycerol) were characterized by their physicochemical properties. pH was recorded using a pH meter (Accumet AE150, Fisher Scientific, Ottawa, ON, Canada) at 25 °C. The viscosity test was performed using a viscometer (LVDVE115, Brookfield Engineering Laboratories Inc., Middleboro, MA, USA) at 25 °C. The conductivity test was recorded using a conductivity meter (Model STAR A2120, Thermo Scientific, Beverly, MA, USA) at 25 °C. Density was measured (g/mL) by calculating the mass (g) and volume (mL) at ambient temperature (23 °C).

3.3.2.5. Optimization of protein extraction variables based on solubility

Optimization was carried out according to the design matrix shown in Table 3.2. Approximately 1 g of fava bean flour was transferred to a 50 mL Falcon tube containing DES with the appropriate optimization parameters. The contents of the tubes were thoroughly vortexed for 10-15 sec, followed by homogenization (Model 850, Fisher Scientific, Ottawa, ON, Canada) for 2

min at 20000 rpm. Then, extraction was performed using a shaking water bath (200 rpm) at the pre-determined temperature and time. After extraction, tubes were allowed to cool at room temperature (~22 ºC), centrifuged (4 ºC, 1507 g, 10 min), and decanted for protein quantification by using Bradford protein assay to determine the protein concentration at 595 nm using UV-spectrophotometer (PerkinElmer FL 6500, Shelton, CT, USA)**.** Bovine serum albumin (BSA) was used to develop a standard curve to calculate the protein content.

After optimizing five variables, the effect of pH on protein extraction was studied using a single-factor experiment. pH was adjusted using 0.1 M NaOH and 0.1 M HCl in the pH range of 3.0-8.0, and extraction was carried out using the same procedure described above while maintaining the optimized variables constant.

3.3.2.6. Determination of protein extraction yield and recovery rate under optimized conditions

Protein extraction was performed using optimized conditions, following the same procedure described in Section 3.2.2.5, except that a circulation water bath (Model 6200 H7, Fisher Scientific Inc., Pittsburgh, PA, USA) with a 3 L jacketed beaker was used. After extraction, the supernatant was dialyzed using a 3.5 kDa molecular weight cut-off (MWCO) dialysis membrane for 5 days at 4 °C, with water changes every 4 hours. The resulting protein solution was collected, freeze-dried, and stored at -20 ºC. The extracted protein yield and recovery rate were calculated using equations (3) and (4).

$$Protein\ yield\ \%\ (DW) = (Ep\ \times P)/Rp\ \times 100 \quad (3)$$

$$Protein\ recovery\ rate\ \%\ (DW) = \ (Ep\ \times P)/Rw\ \times 100 \quad (4)$$

Where *Ep* is the Weight (g) of extracted protein on a dry weight (DW) basis, *P* is the percentage of purity of extracted protein in DW, *Rp* is Protein content (g) in initial raw material on a DW basis, *Rw* is the weight (g) of initial raw material on DW basis.

3.3.2.7. Conventional alkaline protein extraction

Conventional alkaline extraction followed by isoelectric precipitation was carried out according to the methods described by Karaca et al. (2011) with slight modifications. First, ground fava bean flour was dispersed in Milli-Q water at a 1:10 (w/v) solid: liquid ratio, thoroughly mixed for 10-15 sec, and homogenized (Model 850, Fisher Scientific, Ottawa, ON, Canada) at 14,000 rpm for 2 min. The dispersion was adjusted to pH 9.5 using 5 M NaOH, and protein was extracted at room temperature (22 ºC, 200 rpm, 2 hrs) followed by centrifugation (17,709 g, 20 min, 4 ºC). Then, the supernatant was adjusted to pH 4.5 using 1 M HCl solution. The precipitated protein was centrifuged as above to collect the protein pellets, followed by washing twice with Milli-Q water. Finally, the protein pellets were dispersed in Milli-Q water, adjusted to a pH of 7.0, freeze-dried, and stored at -20 °C.

3.3.2.8. Proximate analysis

The moisture content of the samples was determined by drying at 105 ºC (Thermo Fisher Scientific, Pittsburgh, PA, USA) for 6 hrs until a constant weight was achieved, while the crude protein content was analyzed using the Kjeldahl method (AOAC, 2000). The total nitrogen content was converted to crude protein using the factor 6.25 (Karaca et al., 2011). The ash content was analyzed at 550 ºC overnight using a Model 1100 box furnace (Thermo Scientific, NC, USA), and the crude fat content was determined by the Soxhlet method (Nielsen, 2010). The total starch (AOAC 996.11) content was analyzed using the Megazyme enzyme assay kit (Megazyme, Ireland). All determinations were performed in triplicate to generate statistically valid data.

3.3.3. Characterization of extracted protein fractions and raw material

3.3.3.1. Particle size distribution

Particle size distribution of fava bean flour and extracted protein was characterized according to the method described by Alonso-Miravalles et al. (2019) with slight modification, using static laser light diffraction (Mastersizer 2000, Malvern Instruments Ltd, Malvern, UK). Samples were mixed separately with Milli-Q water at a concentration of 1% protein (w/v), adjusted to pH 7.0, and stirred for 48 hrs at 4 ºC to create well-dispersed samples. The dispersions were equilibrated at room temperature (~22 ºC) and introduced into the dispersing unit using Milli-Q water as dispersant by setting the refractive index, absorption index, and dispersant refractive index of protein and fava bean flour at 1.45, 0.1 and 1.33, respectively, until a laser obscuration achieved in the range of 12%. The results were reported as the volume-weighted mean particle diameter (D 4,3), surface-area-weighted mean particle diameter (D 3,2), and volume percentiles Dv (0.1), Dv (0.5), and Dv (0.9).

3.3.3.2. Changes in protein secondary structures

Fourier-transformed infrared spectroscopy (FTIR) was employed to characterize the secondary structure changes of extracted protein isolates using a Nicolet 6700 FTIR spectrometer (Thermo Electron Co., WI, USA). IR spectra in the 400-4000 cm^{-1} range were collected at a resolution of 4 cm^{-1} using 120 scans. The collected spectra were normalized, and the amide I peak (1600-1700 cm^{-1}) was located to generate the second derivative curve after subtracting the baselines. The second derivative curve was generated using the Savitzky-Golay smooth function, with a window size of 9, in the Origin 2021 software (OriginLab Corporation, MA, USA). The multiple peak fitting of the second derivative curve was then performed using the Gaussian function to identify the protein's secondary structure components with the same software as above.

3.3.3.3. Morphological characteristics

The morphological characteristics of the protein isolates and fava bean flour were examined using scanning electron microscopy (SEM) (Quanta 650 FEG, FEI Company, OR, USA) coupled with an Everhart-Thornley (ET) detector. Before microscopic analysis, the samples were coated with gold-palladium (Au-Pd) using a sputter coating unit (50 mTorr pressure, 45 mA, 60 sec) and dried under nitrogen purging (Denton vacuum desk II, Denton Vacuum Inc., NJ, USA). The microscopic images were analyzed using xT microscope control (ver. 6.2.8) software (Quanta 650 FEG, FEI Company, OR, USA).

3.3.3.4. Color profile

The color profiles of the extracted protein isolates and flour were characterized using a color spectrophotometer (CM-3500d, Minolta Co., Ltd., Osaka, Japan). The sample was placed on the target mask in a Petri dish, and reflectance was measured at a 2 ° standard observer and with Illuminants based on *L*, a**, and *b** values on the Hunter scale.

3.3.3.5. SDS-PAGE analysis of fava bean flour and extracted proteins

The polypeptide composition of the extracted protein isolates was characterized using SDS-PAGE, as described by Laemmli (1970) and Raikos et al. (2014), with a slight modification using a Mini-Protein electrophoresis unit (Bio-Rad Laboratories Inc., CA, USA). Protein samples were dispersed in 5% sodium dodecyl sulfate solution, heated at 85 °C for 1 hrs, and centrifuged (12,310 g, 10 min) to give a 4 mg/mL (w/v) protein concentration. Then, an equal volume of Laemmli buffer and sample was mixed to obtain 2 mg/mL (v/v) protein concentration. For the reducing condition, 2-mercaptoethanol was added to the sample buffer. The samples were then heated at 90 °C for 5 min. An aliquot of 5 µL of each prepared sample and 10 µL of Precision Plus protein standard were loaded into a 4-15% Mini-Protean TGX precast gel. The gels were run at

150 V for 1 hrs in a Mini-Protean II electrophoresis cell. The gels were stained with Coomassie Brilliant blue R-250 for 1 hrs, followed by destaining with 50% methanol and 10% acetic acid in water for 2 hrs. The resulting protein bands were imaged using the Bio-Rad ChemiDocTM imaging system (Bio-Rad Laboratories Inc., CA, USA).

3.3.3.6. Amino acid analysis

Samples were prepared according to AOAC Official Method 982.30, with 6 N hydrochloric acid hydrolysis over 24 hrs for the measurement of most amino acids (AOAC, 1995), except for the sulfur-containing amino acids methionine and cysteine, and tryptophan. Methionine and cysteine were analyzed according to AOAC Official Method 985.28, where proteins were first oxidized with performic acid prior to acid hydrolysis, as described above. For both hydrolysis sets, the amino acids were derivatized and separated using an AccQ-Tag Ultra C18, 1.7 μm column (AccQ-Tag Ultra system, Waters Ltd., Mississauga, ON) on a Shimadzu UPLC system, complete with an SIL-30 AC autosampler. For tryptophan, samples were subjected to alkaline hydrolysis and analyzed using ISO protocol 13904 (International Organization for Standardization, 2016). For quality control, the NIST soy flour Standard Reference Material 3234 was used. The hydrated molecular weights of all amino acids were used for quantification, and the data were expressed on a percentage by weight basis

3.4. Results and Discussion

3.4.1. Optimization of protein extraction and experimental validation

The protein extraction levels, as affected by the independent variables, are shown in Table 3.2. Fava bean protein yield varied from 37.47 ± 2.28 mg / g to 103.38 ± 2.31 mg / g DW. The lowest yield (37.47 ± 2.28 mg /g) was found in 100% DES. In contrast, the highest yield (103.38 ± 2.31 mg/g) was achieved in the DES solvent system containing 30% (w/w) water, indicating the

importance of adding water to the DES solvent system to improve protein extraction efficiency. The protein extraction yield was optimized using the RSM model with a second-order polynomial equation (Equation 5), which shows an empirical relationship between protein yield and independent variables.

$$\text{Protein yield (mg/g DW)} = 28.3 + 29.65X1 + 1.54X2 - 0.166X3 - 25.8X4 + 5.009X5 - 1.160X1X1 - 0.0744X2X2 - 0.2105X3X3 + 5.10X4X4 - 0.02773X5X5 - 0.6230X1X5 + 0.0696X2X3 - 0.972X2X4 + 0.385X3X4 \quad (5)$$

Based on the analysis of variance (ANOVA) table (Table 3.1S, Appendix 1), the generated data showed a significant model ($P < 0.000$) with R^2, R^2 adjusted, and R^2 predicted values of 0.9854, 0.9727, and 0.9468, respectively. The high value of R^2 (> 0.8) suggests that the generated polynomial model was well-fitted with the experimental data. The insignificant lack of fit (0.593, $P > 0.05$) indicated the adequacy of the model in predicting protein yield under the selected experimental conditions. The quadratic model generated four linear terms (X_1, X_2, X_3 and X_5), five square terms (X_1X_1, X_2X_2, X_3X_3, X_4X_4, X_5X_5) and four interaction terms (X_1X_5, X_2X_3, X_2X_4, X_3X_4), which were significant ($P < 0.05$). The significant interaction terms are shown in Figure 3.1, along with the interaction plot and corresponding contour plots obtained from the model related to protein yield. The effect of liquid: solid ratio and temperature on protein yield is illustrated in Figures $3.1a_1$ and $3.1a_2$, which indicate that the protein extraction yield increased (> 84 mg/g DW) with an increase in the liquid: solid ratio from 10:1 to 28:1. Increasing the amount of DES could facilitate the diffusion of more solvents into the matrix, promoting better protein solubility. However, the protein yield began to decrease with a further increase in the liquid: solid ratio up to 30. This may be because all soluble proteins are already extracted into the DES system. Increasing the temperature of DES can reduce its viscosity, thereby promoting the mass transfer rate and enhancing the extraction process. However, in this study, the yield decreased when the extraction

temperature increased beyond 50 ºC up to 90 ºC. This could be attributed to the denaturation of proteins at higher temperatures, which breaks the intermolecular interactions of proteins. Furthermore, at higher temperatures, DES components can dissociate, disrupting the hydrogen bond network formed within protein molecules. Consequently, this can lead to a reduction in the solubility of proteins. Therefore, according to the generated model, the protein extraction yield was maximized at an extraction temperature of 50 ºC and a liquid: solid ratio of 28.

Figures $3.1b_1$ and $3.1b_2$ represent the effect of the interaction between the ChCl: Gly molar ratio and percent water content (w/w) on protein extraction yield. It was observed that there was a positive correlation between water content and the DES molar ratio in terms of protein yield. Replacement of DES with water could result in a gradual weakening of the distinct hydrogen bond network between ChCl and Gly (Vilková et al., 2020). Additionally, the excess Gly could weaken the internal hydrogen bond network when the molar ratio of ChCl and Gly is increased from 2 to 6 (Chen et al., 2021). These interactions may facilitate mass transfer within the extraction phase, resulting in higher extraction efficiency. Interestingly, at a water content of 0-30% w/w, the extraction yield increased drastically when the molar ratio increased from 2 to 6. However, above 30% (w/w) water substitution in the DES system, protein yield reached a maximum with 40% (w/w) water content, which was independent of the molar ratios studied. This indicates that the water content of DES has a profound impact on the behavior of its components, which can affect the protein extraction yield. It has been demonstrated that the addition of water below 50% (w/w) can solvate the supramolecular microscopic structure of DES with partial disruption and modification of its molecular arrangement while preserving the properties of DES (Vilková et al., 2020). In this study, the addition of water from 30 to 40% (w/w) likely resulted in partial dissociation of the ChCl and Gly components, promoting hydrogen bond formation between the

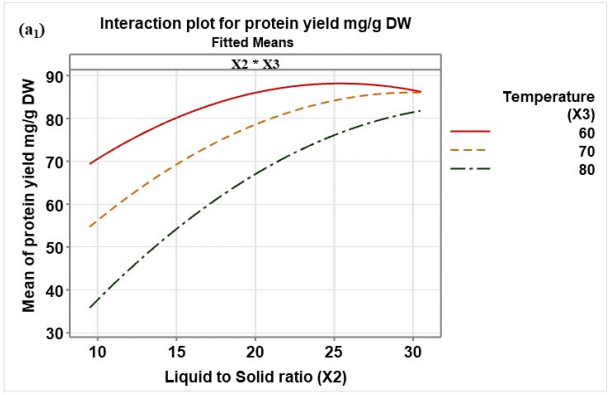
Cl^- anion of ChCl and water molecules. This might weaken intermolecular interactions between ChCl and Gly, hence minimizing the diffusion of protein molecules into the solvent phase. At this point, even though Gly content is increased, it reduces the proportion of ChCl in the whole system, affecting interactions between proteins and ChCl. Therefore, the results suggest that increasing the molar ratio from 2 to 6 showed a negligible impact on the protein yield at the 30-40% (w/w) water addition. However, further investigations are required to understand the impact of water substitution on the ChCl and Gly DES system in relation to protein extraction.

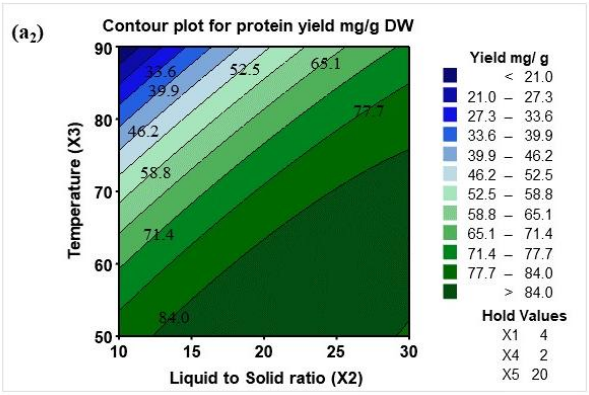
Table 3.2. RSM design matrix for the optimization of fava bean protein extraction using DES

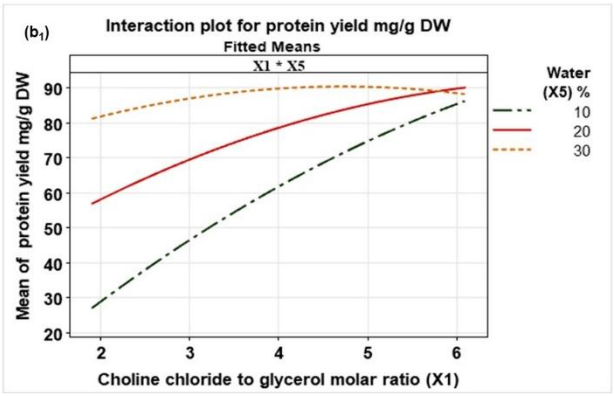
Run Order	X_1	X_2	X_3	X_4	X_5	Extraction yield mg/g DW
1	4	20	70	2	40	98.17 ± 2.99
2	6	20	70	2	20	90.03 ± 0.60
3	4	20	70	1	20	82.54 ± 1.56
4	5	15	60	2.5	30	94.79 ± 1.64
5	4	30	70	2	20	87.14 ± 0.73
6	4	20	70	2	20	79.96 ± 3.24
7	5	25	60	1.5	30	103.38 ± 2.31
8	3	15	60	2.5	10	50.48 ± 0.40
9	4	20	70	3	20	85.49 ± 0.77
10	4	20	90	2	20	49.74 ± 2.73
11	5	15	60	1.5	10	78.16±6.99
12	4	20	70	2	20	77.93 ± 0.53

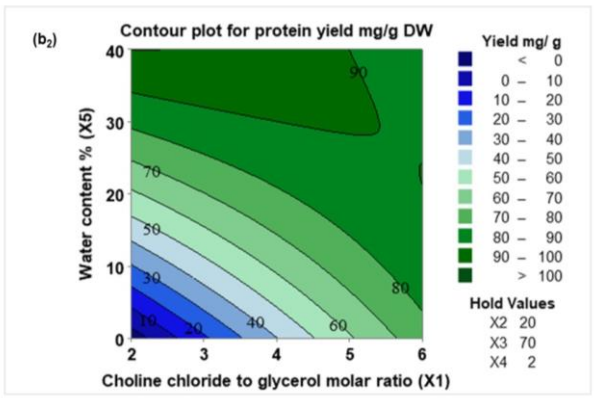
13	3	15	60	1.5	30	85.06 ± 2.58
14	4	20	70	2	20	74.23 ± 1.20
15	3	25	60	1.5	10	59.96 ± 0.76
16	5	25	80	1.5	10	73.82 ± 3.07
17	3	15	80	2.5	30	73.27 ± 4.06
18	5	25	60	2.5	10	83.67 ± 5.07
19	5	15	80	2.5	10	54.40 ± 4.08
20	4	20	50	2	20	91.25 ± 1.57
21	3	25	80	1.5	30	84.38 ± 2.44
22	3	15	80	1.5	10	42.15 ± 1.33
23	4	20	70	2	20	76.79 ± 1.74
24	2	20	70	2	20	58.52 ± 1.85
25	3	25	80	2.5	10	48.46 ± 2.83
26	5	15	80	1.5	30	62.48 ± 4.94
27	5	25	80	2.5	30	87.75 ± 5.71
28	4	20	70	2	20	82.20 ± 4.53
29	3	25	60	2.5	30	92.12 ± 1.16
30	4	10	70	2	20	55.81 ± 0.69
31	4	20	70	2	20	80.18 ± 2.72
32	4	20	70	2	0	37.47 ± 2.28

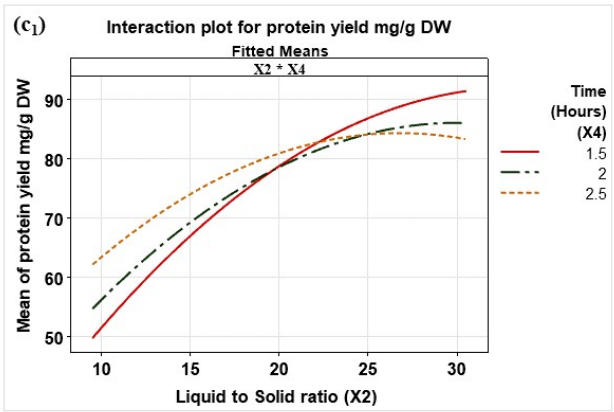
Optimization of fava bean protein extraction using RSM consisting total of 32 runs with 6 center points with 5 variables: X_1 - ChCl: Gly molar ratio (w/w), X_2 - liquid: solid ratio (w/w), X_3 - extraction temperature (°C), X_4 –extraction time (hrs) and X_5 – water content (w/w). The response variable, protein yield mg / g (DW), is presented as mean ± Standard deviation (n=3). DES- Deep eutectic solvent.

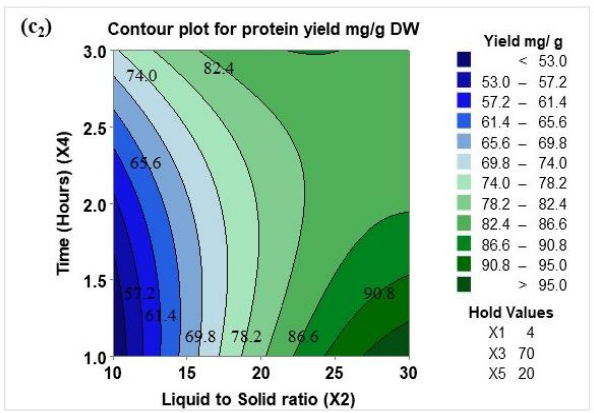












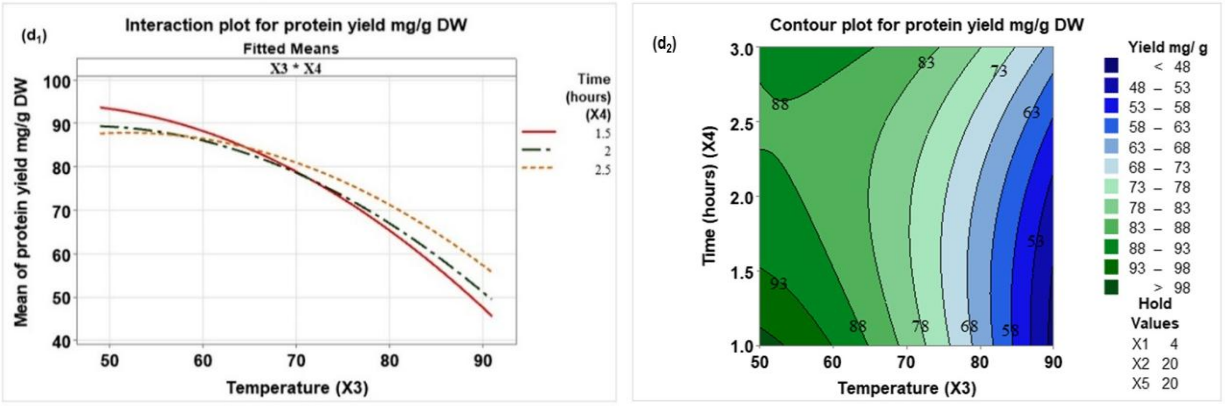


Figure 3.1. Interaction terms obtained from the polynomial equation of the RSM model with $P < 0.05$ and lack of fit $P > 0.05$ showing interaction and contour plots **(a_1), (a_2)** for the effect of liquid :solid ratio (w/w) versus temperature (°C), **(b_1), (b_2)** for the ChCl: Gly molar ratio (w/w) versus water content % (w/w), **(c_1), (c_2)** for the liquid: solid ratio (w/w) versus time (hrs), and **(d_1), (d_2)** for the temperature (°C) versus time (hrs), on protein yield (mg/g DW) in DES protein extraction from fava bean.

Moreover, as shown in Figures 3.1c_1 and 3.1c_2, a positive correlation existed between the liquid: solid ratio and extraction time on the protein yield. This is because increased extraction time allows for more contact between the raw material and DES, thereby improving protein solubility and extraction efficiency. Nevertheless, protein yield started to decrease when the liquid: solid ratio exceeded 28. Therefore, it is noteworthy that a lower extraction time resulted in a higher protein yield at a liquid: solid ratio of 28. As previously explained, this can be attributed to the enhanced solubility and extraction of proteins into the DES at a higher liquid-to-solid ratio, which promotes higher solubility during shorter extraction times. Therefore, the maximum protein yield was obtained at an extraction time of 1 hrs and a liquid: solid ratio of 28:1. Furthermore, a negative correlation was observed between extraction temperature and time on the protein yield,

as shown in Figures 3.1d$_1$ and 3.1d$_2$. When the temperature was increased from 50 to 90 °C with an extended extraction time, the protein yield decreased gradually. Denaturation of proteins at elevated temperatures can result in a lower yield of soluble proteins, thereby affecting the protein extraction process. Therefore, the extraction yield at 1 hrs and 50 ºC was shown to be the optimum conditions for generating a maximum protein yield. Overall, the optimum protein extraction conditions for obtaining the maximum protein yield, as determined by the RSM model, were, 1:2.04 (w/w) ChCl: Gly molar ratio, 27.17:1 (w/w) liquid: solid ratio, temperature of 50 ºC, extraction time of 1 hrs, and a water content of 40% (w/w). In order to simplify the solvent synthesis, the liquid: solid ratio and ChCl: Gly ratio were adjusted to 28:1 and 1:2, respectively. The optimum predicted protein yield was experimentally validated using optimized protein extraction conditions. Table 3.2S (Appendix 2) represents the response prediction and validation of the protein extraction conditions. The experimental protein extraction yield at optimal conditions was 124.48 ± 0.91 mg/g DW, comparable to the predicted protein yield of 125.30 mg/g DW obtained from the model. Thus, the generated second-order polynomial mathematical model (Equation 5) confirmed suitability for predicting protein extraction yield from fava bean in this study. Lin et al. (2020) optimized protein extraction from Bamboo shoots using the same RSM model, but with a ChCl and levulinic acid DES-based system. However, the protein yield achieved (35.10 mg/g DW) was significantly lower than the value obtained in our study. An extraction yield of 0.3462 ± 0.046 g / g was recently reported for soybeans following the same optimization method using ChCl and Gly DES system (Chen et al., 2021); this value is higher than in previous studies and our results. Differences in the raw material's protein content, DES components, and their molar ratio, as well as the water content in DES, and extraction time and temperature, may be some of the factors responsible for variations in protein extraction yield.

3.4.2. Characterization of physicochemical properties of DES

The physicochemical properties of DES are presented in Table 3.4S (Appendix 4). The high viscosity of DES is a potential barrier to industrial application. Therefore, the hydration of DES is imperative to tailor its properties and enhance its applicability in protein extraction (Yu et al., 2021). Water is amphoteric and can accept and donate hydrogen bonds (Kaur et al., 2020). Thus, water can potentially disrupt DES's microstructure and hydrogen bond network. As a result, the physicochemical properties of DES could be altered. This is clear in the results obtained in our study (Table 3.4S-Appendix 4). The pH is an important consideration as DESs are formed using a mixture of Lewis or Bronsted acids and bases. According to Table 3.4S, both pure DES and the DES with 40% (w/w) water exhibited slightly acidic pH. The acidity of DES is primarily determined by the type of hydrogen bond donor (HBD) (Kareem et al., 2010; Skulcova et al., 2018). Since Gly (trihydric alcohol) has acidic hydrogens in its structure, it is probably responsible for contributing to the slight acidity of the solvent (Skulcova et al., 2018). After the addition of water up to 40% (w/w), the pH of DES decreased significantly from 5.84 ± 0.02 to 5.25 ± 0.02. Water could act as a hydrogen bond donor, further decreasing the pH of the solvent. The viscosity of the pure DES was 329.80 ± 1.85 cP, which is comparable to the reported data (Bryant et al., 2021; Zhang et al., 2012). The high viscosity of DES is often attributed to the extensive network of hydrogen bonds between components (Yu et al., 2021). A drastic decrease in viscosity could occur upon adding water due to the gradual weakening of hydrogen bond interactions (Dai et al., 2015). Our results show a significant reduction of viscosity (from 329.80 ± 1.85 to ~9.43 ± 0.12 cP) after adding water. As a result, an increase in conductivity (from 0.613 ± 0.02 to 17.38 ± 0.16 ms/cm) was observed in the 40% water-substituted DES. In contrast to our results, Van Osch et al. (2019) reported a slight reduction in the viscosities of hydrophobic DESs after the addition of

water from 200 to 1100 ppm. Another important physical property is density, which provides information about the intermolecular interactions between DES components. The density of ChCl- and Gly-based pure DES often ranges from 1.18 to 1.20 g/cm³ (Bryant et al., 2021; Zhang et al., 2012), which aligns with our results. The density also showed a significant reduction from 1.20 ± 0.001 to 1.14 ± 0.003 mg/mL with the addition of 40% w/w water. Dai et al. (2015) demonstrated that adding a range of water to some natural DES (NADES) could increase conductivity up to 100 times. Further, the author revealed that both density and viscosity decreased significantly as the water content increased. Our results suggest that the optimized DES system with 40% (w/w) water has a significant impact on the solvent's physicochemical properties, which favors protein extraction. Thus, the addition of water can significantly reduce costs in industrial applications and tailor these properties to a wide range of plant protein extractions.

3.4.3. Effect of pH on protein extraction

The protein yield extracted under validated optimum extraction conditions, ranging from pH 5.0 to 8.0, is presented in Table 3.3S **(**Appendix 3**).** The pH range was chosen considering two factors: the isoelectric point of fava bean proteins (4.5) and pH conditions that are not at extreme levels. As shown in Table 3.3S (Appendix 3), the extracted yield was not significantly impacted by the pH range studied ($P > 0.05$). This suggests that the ChCl and Gly DES system may create a buffering action in the pH range of 5.0 to 8.0. The buffering action of DES offers additional advantages, as protein extraction can be performed near neutral pH without adjustment. Therefore, in our study, protein extraction was carried out using optimized conditions without adjusting the pH for further analysis. Similar to our study, previous studies on oats (Yue et al., 2021), bamboo shoots (Lin et al., 2020), oilseed cakes (Grudniewska et al., 2018), and brewer's spent grains (Wahlström et al., 2017) have also reported protein extraction without pH adjustment. The type of

HBD in DES has a significant influence on the pH (Skulcova et al., 2018), and the addition of water may also have substantial effects on the behavior of DES components, altering the pH during the extraction process. Therefore, further in-depth investigations are required to understand the interactions between DES components on protein extraction.

3.4.4. Protein content, yield, and recovery rate of extracted protein fractions

Table 3. 3. Protein extraction yield and recovery rate of DES and alkaline extracted proteins

Extraction Method	Protein Content %	Protein yield %	Protein recovery rate %
DES extraction	92.33 ± 2.28^{a}	65.42 ± 6.53^{a}	23.15 ± 2.31^{a}
Alkaline extraction	92.50 ± 1.36^{a}	60.76 ± 1.16^{b}	$21.740.19^{b}$

All data were analyzed by analysis of variance followed by Tukey's test (n=3) and expressed as dry weight basis. Different superscripts within a column indicate a significant difference ($P < 0.05$). DES- Deep eutectic solvent.

The protein extraction yield and recovery rate of DES and alkaline-extracted proteins are shown in Table 3.3. No significant difference was found between the protein content of DES (92.33 ± 2.28%) and that of alkaline-extracted proteins (92.50 ± 1.36%). Protein yield and recovery rate, however, differed significantly between the two extraction methods ($P < 0.05$). Compared with alkaline extraction (60.76 ± 1.16%, 21.74 ± 0.19%), DES extraction exhibited significantly higher protein yield and recovery rates (65.42 ± 6.53%, 23.15 ± 2.3%), respectively. Grudniewska et al. (2018) reported protein contents of 40 - 50% and protein yields of 11.5-19.9 g/100 g and 8.4-34.2 g/100 g from evening primrose cake and rapeseed cake, respectively. It is worth noting that using

extreme temperatures for protein extraction would result in extensive protein denaturation and loss of protein functionality; therefore, careful selection of the extraction temperature is imperative. An extraction yield of 7.98% and a protein content of 55.28% were reported for oats at 80 ºC for 90 min with ChCl-butanediol and its water binary mixture. Furthermore, Chen et al. (2021) reported a protein yield of 0.3462 ± 0.046 g/g using a ChCl and Gly-containing DES system from soybeans, but the protein content of the extract was not reported. Notably, our values were much higher than those reported values, and variations in extraction conditions and raw materials used in our study may have substantially impacted the protein yield and content. Based on our results in this study, we stipulated that the solubility and stability of proteins in the solvent might play a significant role in achieving higher protein extraction efficiency. Also, molecular interactions between DES components and water, as well as protein molecules, could induce improved protein solubility and stability properties. Therefore, the mechanism underlying the enhanced protein extraction efficiency may be associated with both the solvent's intermolecular structure and arrangements, as well as its interactions with proteins. The inner structure of the DES is believed to be formed as a polymer-like matrix (Bryant et al., 2021; Liu, Friesen, McAlpine, et al., 2018). The protein molecules, therefore, can dissolve into the space of the polymer network. However, with pure DES, extraction efficiency was the lowest (Table 3.2), suggesting that the high viscosity of DES could impede the mass transfer within the polymer matrix. Interestingly, with the addition of water at 40% w/w, the DES components ratio may be altered by modifying the inner space (size) of the polymer network. This may facilitate the dissolution of more protein molecules into the DES structure's space, coupled with a decrease in DES's viscosity. Following the DES's structural properties, protein molecules may have become a part of the DES matrix through various types of molecular interactions, primarily by hydrogen bonds and electrostatic and Van der Waals forces

(Hansen et al., 2021; Kaur et al., 2020), thus hydrogen bonding might contribute as the major driving force for the solvation of proteins (Qiao et al., 2021), thereby increase in extraction efficiency. Additionally, based on the ionic strength of the DES solution, the salting-out effect may have played a significant role in achieving the higher extraction efficiency (Hewage et al., 2022). However, further studies are warranted to elucidate the exact mechanism of protein extraction using DES.

3.4.5. Proximate composition

The proximate analysis of extracted protein fractions and fava bean flour is presented in Table 3.4. The main components of the fava bean flour were starch (54.40 ± 0.77%) and protein (30.68 ± 0.12%). A similar amount of proteins was reported by Mayer Labba et al. (2021) and Millar et al. (2019). The proteins obtained from DES and alkaline extraction had higher protein contents of 92.59 ± 2.11% and 92.71 ± 1.33%, respectively. Herein, proteins obtained from both extraction methods can be considered protein isolates: DES-FBPI (Deep eutectic solvent-extracted fava bean protein isolates) and ALK-FBPI (alkaline-extracted fava bean protein isolates). The starch content of DES-FBPI was 0.12 ± 0.06%, but for ALK-FBPI, it was not detected. According to the proximate composition of ALK-FBPI, the calculated total carbohydrate content, determined by the difference method, was approximately 1.15%. Out of this, the starch fraction might be very small, and it is more likely that the minute starch content in the ALK-FBPI was not sensitive enough to be detected by the analyzed method. The fat content in the flour was low (1.73 ± 0.45%), but a slight increase was observed for both DES-FBPI and ALK-FBPI. This may be due to the partial binding of lipids to proteins, which is affected by homogenization. On the other hand, Ash content was not varied among the flour, DES-FBPI, and ALK-FBPI.

Table 3. 4. Proximate composition of extracted protein fractions and fava bean flour

Ingredients	Moisture %	Ash %	Fat %	Protein %	Total starch %
Fava bean flour	13.31 ± 0.02	3.18 ± 0.03	1.73 ±0.45	30.68±0.12	54.40 ± 0.77
DES- FBPI	7.75 ± 0.83	3.35 ± 0.22	2.05±0.79	92.33±2.28	0.12 ± 0.06
ALK- FBPI	1.28 ± 0.44	3.18 ± 0.08	3.17±1.51	92.50±1.36	ND

Proximate composition of DES- FBPI (Deep eutectic solvent-extracted fava bean protein isolates) and ALK-FBPI (Alkaline-extracted fava bean protein isolates). Results are presented as mean ± standard deviation (n=3) on a dry weight basis. ND- Not detected.

3.4.6. Physiochemical characterization of extracted protein fractions and raw material

3.4.6.1. Particle size distribution

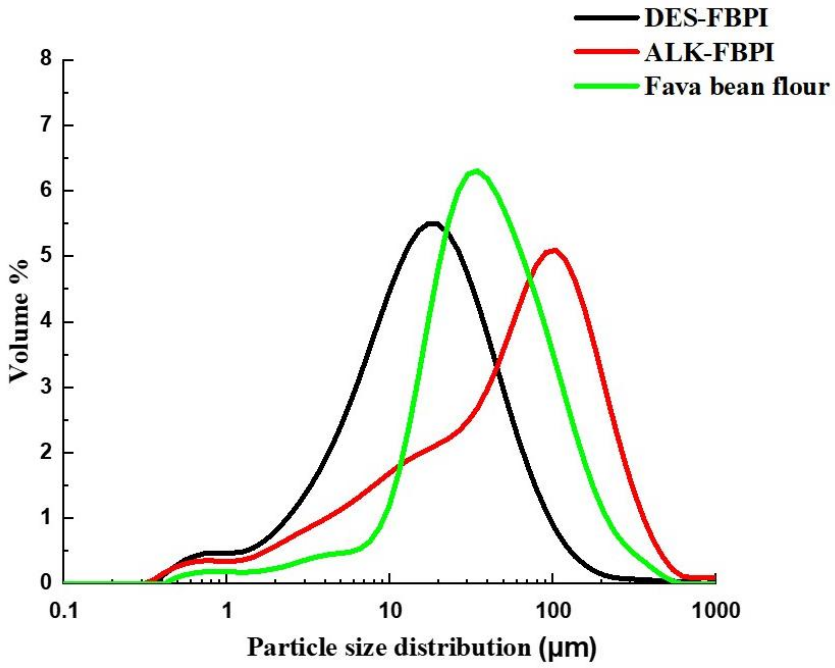


Figure 3. 2. Particle size distribution of DES-FBPI- (Deep eutectic solvent-extracted fava bean protein isolates), ALK-FBPI- (Alkaline-extracted fava bean protein isolates), and fava bean flour.

The volume-weighted particle size distribution of the extracted protein fractions and fava bean flour dispersions is shown in Figure 3.2 and Table 3.5. The particle size distributions of both extracted protein fractions and fava bean flour showed a monomodal distribution in the range of ~0.5 to 400 μm. Our results for fava bean flour were comparable to those of Petitot et al. (2010), who reported a higher amount of fine particles (0-50 μm) and a lower amount of larger particles (160–1000 μm) in fava bean flour. Both volume-weighted mean particle diameter (D [4,3]) and surface area-weighted mean particle diameter (D [3,2]) significantly varied ($P < 0.05$); DES-FBPI exhibited the lowest D [4,3] (28.08 ± 5.80 μm) and D [3,2] (7.64 ± 1.04 μm) compared to fava bean flour (61.56 ± 1.52, 18.16 ± 0.60 μm) and ALK-FBPI (98.87 ± 1.3, 11.52 ± 1.95 μm), respectively. Significantly lower Dv (0.5) and Dv (0.9) were also observed with DES-FBPI; however, no significant differences were found between DES-FBPI and ALK-FBPI for Dv (0.1). Overall, this suggests that DES-FBPI had a significantly greater prevalence of smaller-sized and finer particles compared to ALK-FBPI. Even though ALK-FBPI exhibited the largest particle size compared to fava bean flour and DES-FBPI, 90% of the particles were smaller than 225 μm.

Table 3. 5. Particle size distribution of extracted protein fractions and fava bean flour

Particle size distribution (μm)	Fava bean flour	DES- FBPI	ALK- FBPI
D [4,3]	61.56 ± 1.52^{b}	28.08 ± 5.80^{c}	98.87 ± 1.31^{a}
D [3,2]	18.16 ± 0.60^{a}	7.64 ± 1.04^{c}	11.52 ± 1.95^{b}
D_v (0.1)	13.18 ± 0.53^{a}	3.83 ± 0.84^{b}	5.71 ± 1.88^{b}

D_v (0.5)	41.14 ± 1.72^{b}	17.61 ± 2.57^{c}	65.91 ± 1.63^{a}
D_v (0.9)	132.51 ± 0.86^{b}	58.77 ± 12.63^{c}	224.63 ± 4.67^{a}

Particle size distribution of DES-FBPI- Deep eutectic solvent-extracted fava bean protein isolates and ALK-FBPI- Alkaline-extracted fava bean protein isolates. D [4,3]: Volume-weighted mean particle diameter, D [3,2]: Surface-area-weighted mean particle diameter, Dv (0.1): 10th volume percentile, Dv (0.5): 50th volume percentile, Dv (0.9): 90th volume percentile. All data were analyzed using analysis of variance, followed by Tukey's range test (n = 3). Different superscripts within a row indicate a significant difference ($P < 0.05$).

3.4.6.2. Color

Table 3. 6. Color changes in extracted protein fractions and fava bean flour

Ingredient	*L**	a*	b*
Fava bean flour	88.85 ± 0.00^{a}	-3.40 ± 0.00^{a}	14.16 ± 0.00^{a}
DES- FBPI	75.53 ± 0.08^{b}	1.34 ± 0.04^{b}	16.45 ± 0.14^{b}
ALK-FBPI	76.06 ± 0.13^{b}	1.63 ± 0.06^{b}	25.27 ± 0.55^{c}

Color comparison of DES- FBPI (Deep eutectic solvent-extracted fava bean protein isolates) and ALK- FBPI (Alkaline-extracted fava bean protein isolates). *L** represents lightness, and a* and b* are chromaticity coordinates. All data were analyzed by analysis of variance followed by Tukey's test (n=3). Different superscripts within a column indicate a significant difference ($P < 0.05$).

Color values, as presented in Table 3.6, show a significant ($P < 0.05$) decrease in lightness (*L**) in both DES-FBPI (75.53 ± 0.08) and ALK-FBPI (76.06 ± 0.13) compared to fava bean flour

(88.85 ± 0.00). However, no significant difference in *L** values was observed between DES-FBPI and ALK-FBPI. The decrease in lightness may be attributed to the enzymatic and non-enzymatic oxidation of polyphenolic compounds present in fava beans, such as phenolic acids, flavonols, flavanols, and proanthocyanidins (Sharan et al., 2021). Grudniewska et al. (2018) reported that DES-extracted proteins from rapeseed and evening primrose cake, heated at 60 ºC and 100 ºC, respectively, produced a light beige color, whereas a dark brown color was observed at 140 ºC. Similar observations for soybeans have also been reported (Chen et al., 2021). The fava bean seeds used in our study were dehulled and tannin-free varieties. The DES extraction at 50 ºC likely contributed to the lighter color in the protein isolates (Figure 3.3). Moreover, significant yellowness (b*) was observed for all three ingredients; ALK-FBPI had significantly higher yellowness (25.27 ± 0.55), followed by DES-FBPI (16.45 ± 0.14) and fava bean flour (14.16 ± 0.00). This may be related to the β-carotene present in fava beans, which is relatively stable at high pH levels compared to low pH levels (Qian et al., 2012). From the food application perspective, light-colored proteins have a higher potential for novel food formulations. Therefore, DES extraction offers a promising technology for extracting fava bean proteins.

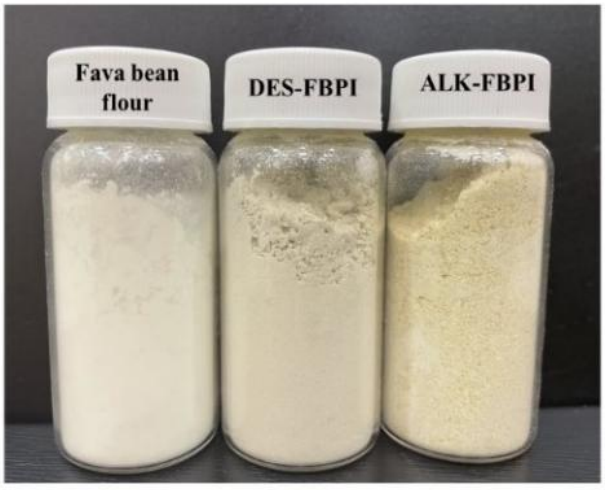


Figure 3. 3. Color comparison of DES- FBPI (Deep eutectic solvent-extracted fava bean protein isolates) and ALK- FBPI (Alkaline-extracted fava bean protein isolates).

3.4.6.3. Morphological characteristics

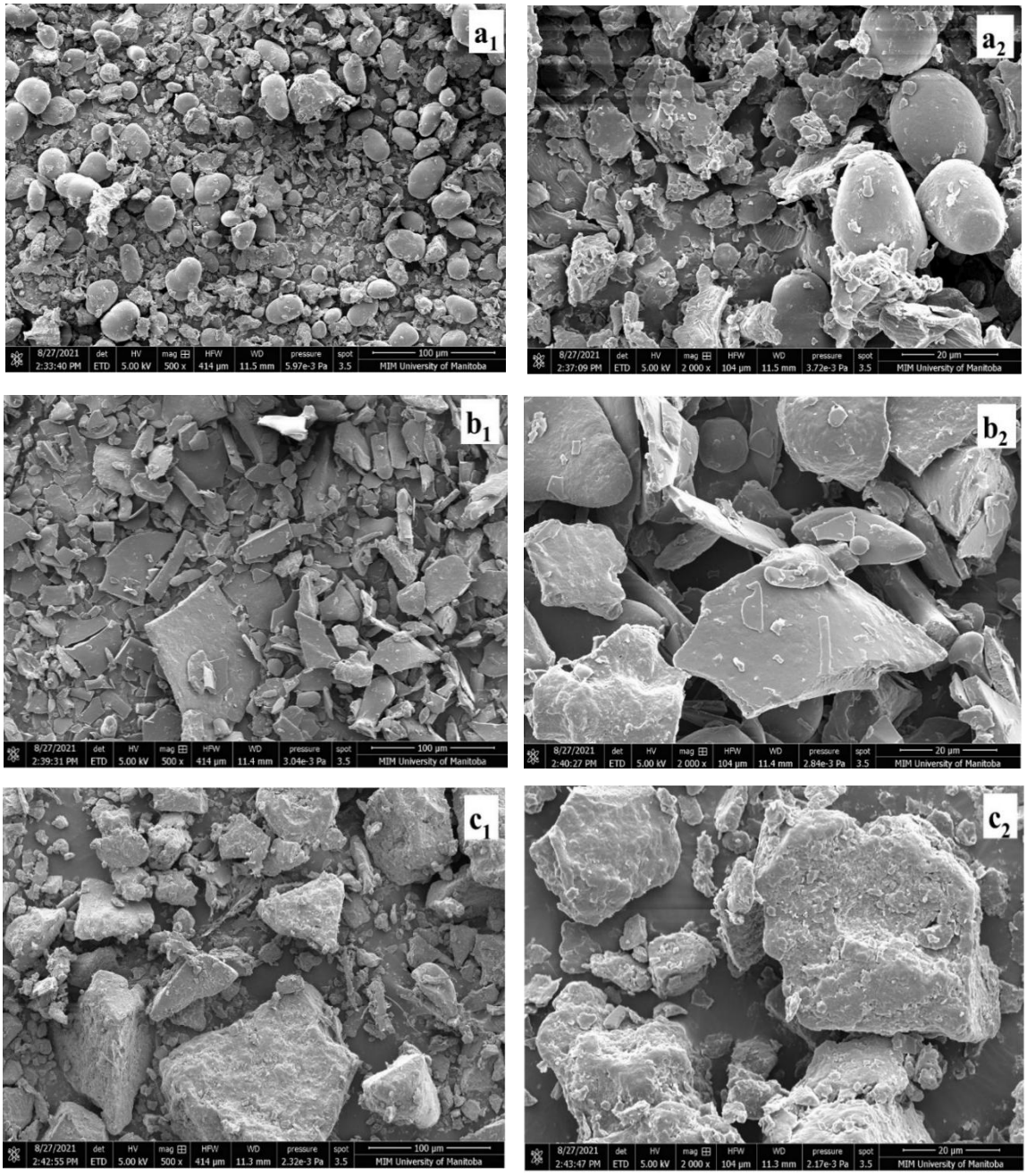


Figure 3. 4. Morphological characteristics of fava bean flour and protein fractions. (**a_1 and a_2**); fava bean flour, (**b_1 and b_2**); DES-FBPI (Deep eutectic solvent-based extracted fava bean protein

isolates), and (**c_1 and c_2**); ALK-FBPI (Alkaline-extracted fava bean protein isolates) at 500× and 2000× magnification, respectively.

Figure 3.4 shows the microscopic images of extracted protein fractions and fava bean flour. Fava bean flour was characterized by large, rounded, and elliptical starch granules surrounded by smaller protein bodies (Figures 3.4a_1 and 3.4a_2). Our observation is in agreement with the findings of De Paiva Gouvêa et al. (2023). The characteristic morphology of DES-FBPI (Figures 3b_1 and 3b_2) and ALK-FBPI (Figures 3c_1 and 3c_2) revealed an irregular, sheet-like structure, which was attributed to the freeze-drying effect used for drying the proteins. Similar observations were reported by Sahni et al. (2020) for alfalfa protein isolates and soybean hydrolysates by Wang et al. (2020). DES-FBPI appeared to have a relatively smooth surface and fewer wrinkles compared to ALK-FBPI at higher magnification (×2000). This may be due to the rate of water evaporation during the freeze-drying process. This was evident in the moisture content of ALK-FBPI, which was 1.28% compared to DES-FBPI at 7.75%. Rapid evaporation of moisture can form wrinkles on protein surfaces, giving a rough characteristic, as observed on the ALK-FBPI.

3.4.6.4. Changes in secondary structures

The secondary structure of extracted protein fractions and fava bean flour is presented in Figure 3.5. Changes in secondary structural components were obtained by processing the amide I peak into the second derivative spectra analysis, followed by a peak fitting procedure. Amide I peak (1700-1600 cm^{-1}) originated from C=O stretching vibration of the peptide backbone (80%), and is closely correlated to each secondary structure element (Yang et al., 2015). The amide I peak positions were assigned based on previously published data (Barth, 2007; Haris & Severcan, 1999).

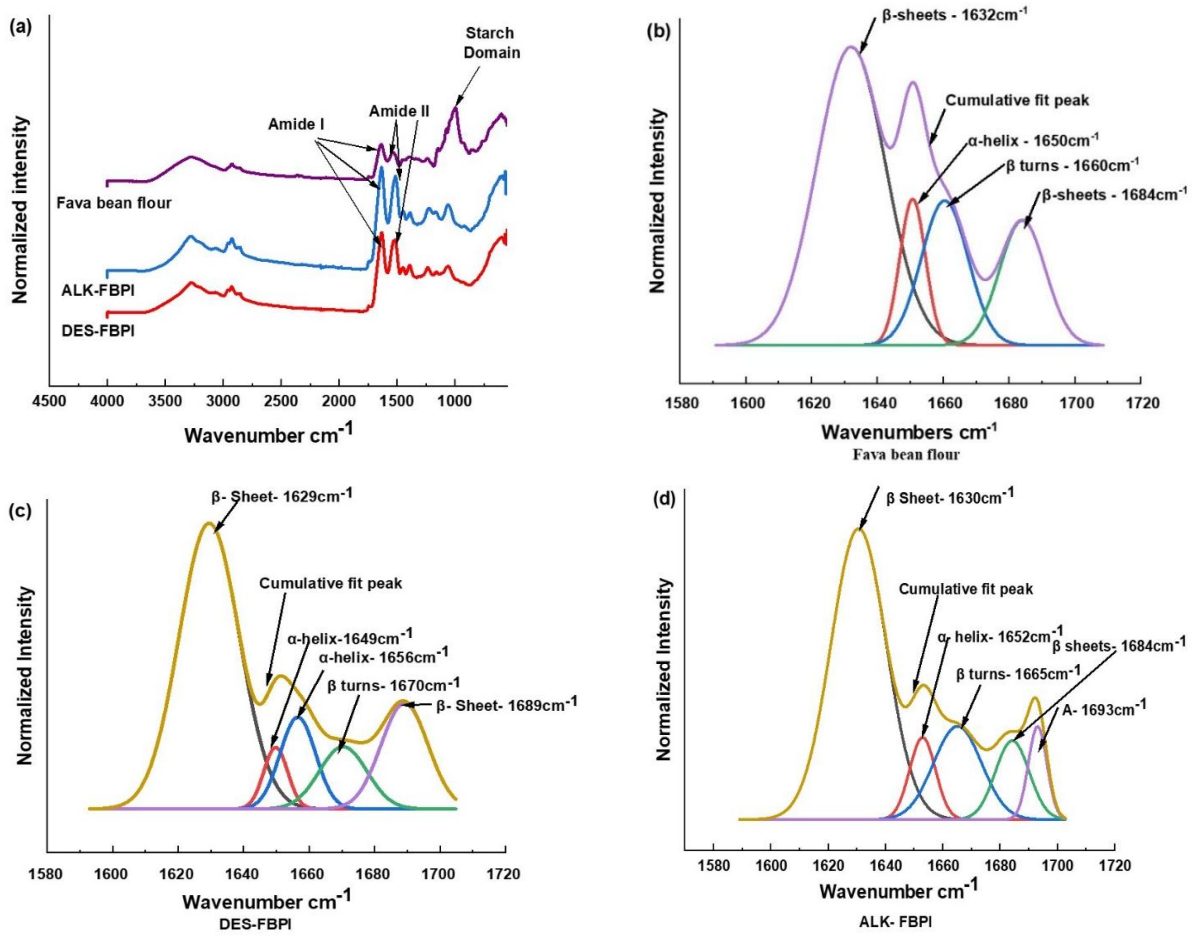


Figure 3. 5. Protein structural changes in extracted protein fractions and fava bean flour. (a) FTIR spectra for fava bean flour, DES-FBPI (Deep eutectic solvent-extracted fava bean protein isolates), and ALK-FBPI (Alkaline-extracted fava bean protein isolates), (b-d) Peak fitting of amide I with the relative proportion of each secondary structure of (b) fava bean flour, (c) DES-FBPI and (d) ALK-FBPI.

As observed in Figure 3.5(a), the amide I peak for flour was less pronounced compared to protein fractions, as starch was the major component (54.40%) in fava bean. The starch domain was prominent at the absorbance band between 1050 and 950 cm^{-1}, which was attributed to C-O-H bending and the α-D-glucopyranose ring structure (Pozo et al., 2018). The spectral contributions of different bands corresponding to β-sheets, α-helix, and β-turns are presented in Figures 3.5(b),

3.5(c), and 3.5(d), and their relative proportions are shown in Table 3.7. The relative proportion of secondary structure components varied depending on the extraction methods. The predominant secondary structure element of the extracted proteins was β-sheets. Most legume seeds have a high amount of β-sheets and a relatively low amount of α-helix (Carbonaro et al., 2012; Shevkani et al., 2019). This is in accordance with our results obtained for fava bean flour. The increased intensity of the band between 1629-1632 and 1684-1689, 1649-1656, 1660-1670, and 1693 cm^{-1} is associated with β-sheets, α-helix, β-turns, and intermolecular β-sheets as aggregates (Barth, 2007; Haris & Severcan, 1999). The primary peak was identified in the amide I region, between 1629 and 1632 cm^{-1}, indicating that the β-sheet structure was dominant for both isolates obtained. A similar trend was reported by Husband et al. (1994) for ALK-FBPI. Both extraction methods decreased the β-sheet content compared to flour. However, a higher β-sheet content (38.61%) was observed in DES-FBPI compared to ALK-FBPI (36.59%), suggesting that alkaline conditions have a greater effect on β-sheet reduction. Similarly, Keivaninahr et al. (2021) observed a reduction in β-sheets for alkaline-extracted fava beans and pea protein isolates. In addition, only ALK-FBPI showed a spectral contribution at the region of 1690-1695 cm^{-1} (7.61%, 1693 cm-1), which is reported to be associated with protein intermolecular aggregates (A) (Keivaninahr et al., 2021; Shevkani et al., 2019a). Also, ALK-FBPI had a slight increase in β-turns (19.71%) compared to fava bean flour and DES-FBPI. Compared to both flour (9.15%) and ALK-FBPI (10.68%), the proportion of α-helix markedly increased in DES-FBPI (21.37%). Our results are comparable to those reported by Yue et al. (2021), who observed an increased α-helix content in oat proteins (45.55%) extracted using a 1:3:1 DES system composed of ChCl: 2,3-butanediol:water. Moreover, Chen et al. (2021) investigated the FTIR spectral differences between commercial and DES-extracted soybean proteins; however, changes in secondary structural components were not

reported. Therefore, further investigations are needed to understand the changes in secondary structure induced by DES protein extraction.

Table 3. 7. Relative proportion of secondary structure of extracted protein fractions and fava bean flour

Secondary Structure Component	Fava bean flour		DES-FBPI		ALK-FBPI	
	Peak center value (cm^{-1})	Relative content (%)	Peak center value (cm^{-1})	Relative content (%)	Peak center value (cm^{-1})	Relative content (%)
β-sheets	1632, 1684	41.04	1629, 1689	38.61	1630, 1684	36.59
α-helix	1650	9.15	1649, 1656	21.37	1652	10.68
β-turns	1660	16.68	1670	17.11	1665	19.71
Intermolecular β-sheets	-	-	-	-	1693	7.61

Secondary structure components analyzed from amide I peak for DES-FBPI (Deep eutectic solvent -extracted fava bean protein isolates, ALK-FBPI (Alkaline-extracted fava bean protein isolates) and fava bean flour.

3.4.6.5. Amino acid composition

The AA profile of fava bean flour and extracted protein fractions (Table 3.8) indicates that aspartic and glutamic acids were the most abundant amino acids, followed by arginine, phenylalanine and tyrosine, and leucine. Tryptophan was the lowest AA, followed by the sulfur-containing AA (SAA), methionine and cysteine. The results are in agreement with previously

published reports (Mayer Labba et al., 2021; Millar et al., 2019; Multari et al., 2015). In the present study, the AA profile of the two extraction methods varied; ALK-FBPI had a higher amount of both essential and non-essential AA content compared to DES-FBPI.

Table 3. 8. Amino acid profile of fava bean flour and extracted protein fractions (g/100g- based on hydrated molecular weight)

Amino acid (AA)	Fava bean flour	DES-FBPI	ALK- PBPI
Essential amino acids (%)			
Leucine	1.950	6.457	7.476
Isoleucine	1.143	3.828	4.308
Valine	1.227	4.034	4.597
Lysine	1.672	5.583	5.746
Methionine + Cysteine	0.479	1.523	1.326
Tryptophan	0.209	0.611	0.790
Histidine	0.426	1.722	1.753
Threonine	0.944	3.019	3.187
Phenylalanine +Tyrosine	2.024	6.974	8.190
Total	10.07	33.75	37.37
Non-essential amino acids (%)			
Serine	1.286	4.314	4.791
Arginine	2.308	7.470	8.760
Glycine	1.102	3.452	3.770
Aspartic acid	2.855	9.303	9.902

Glutamic acid	4.312	14.399	15.721
Alanine	1.037	3.255	3.419
Proline	1.127	3.738	4.133
Total	14.03	45.93	50.50
Acidic AA*	7.167	23.702	25.623
Basic AA**	4.406	14.775	16.259
Acidic: Basic AA	1.63	1.60	1.57
Hydrophobic + uncharged polar AA***	12.528	41.205	45.988

Amino acid profile of DES-FBPI (Deep eutectic solvent-based extracted fava bean protein isolates) and ALK-FBPI (Alkaline-extracted fava bean protein isolates). *- Aspartic + glutamic acid, **- Arginine + histidine + Lysine, ***- Leucine + Isoleucine + valine + Methionine and Cysteine + tryptophan + threonine + phenylalanine and tyrosine + serine + glycine + alanine + proline. Results are presented as one determination (n=1).

However, sulfur-containing AA was higher in DES-extracted proteins, even though these were limiting amino acids, as expected. This could be due to the selectivity of legumin protein fractions during the extraction process. Among the determined AA, acidic (25.623%) and basic AA content (16.259%) in ALK-FBPI were the highest when compared to DES-extracted counterparts (23.702%, 14.775%), respectively. The net charge on the protein surface is determined by the relative ratio of acidic and basic AA, and charged AA are mostly located on the surface of the protein. In DES-FBPI, the net charge of the protein surface was slightly higher (1.60) than that of ALK-FBPI (1.57). In addition, the hydrophobic and uncharged polar AA content in DES-FBPI was lower, at 40.593%, compared to ALK-FBPI (45.988%), suggesting that DES-FBPI

is more hydrophilic in nature. This would be advantageous for functional performance in different food systems.

3.4.6.6. SDS PAGE

SDS-PAGE profile of fava bean flour and extracted proteins under both non-reducing and reducing conditions is presented in Figure 3.6. Based on the electrophoresis profile, characteristic bands of all samples showed large similarities with minor differences. Under non-reducing conditions, prominent band intensities for ALK-FBPI were observed compared to fava bean flour and DES-FBPI; the three prominent bands were attributed to the subunits of globulin; convicilin, legumin, and vicilin fractions with MW of ~66, ~55 and ~49 kDa, respectively (Vogelsang-O'Dwyer et al., 2020). The polypeptide band at a molecular weight of ~74 kDa in all samples may correspond to legumin minor subunits (Sharan et al., 2021). Fava bean legumin subunits are composed of acidic and basic elements linked via intermolecular disulfide bonds (Sharan et al., 2021). Under reducing conditions, legumin was dissociated into two acidic subunits with a molecular weight of ~ 35 kDa (α-legumin) and three basic subunits with a molecular weight of ~ 19-23 kDa (β-legumin) (Żmudziński et al., 2021) in all samples. In addition, the legumin subunit, with a molecular weight of ~ 60 kDa, was also detected in all samples. This indicates that the legumin fraction further disintegrated into its subunits under reducing conditions (Vogelsang-O'Dwyer et al., 2020). However, band intensities corresponding to acidic subunits (~35 kDa) and legumin subunits (~60 kDa) in DES-FBPI were less pronounced than those in fava bean flour and ALK-FBPI. Furthermore, the band visible at ~ 14 kDa, which likely represents the albumin fraction in both fava bean flour and DES-FBPI, was absent in ALK-FBPI. Fava bean protein isolates produced by isoelectric precipitation are typically enriched with globular proteins, as albumin fractions remain soluble at the isoelectric pH (4.5) of globulin and are lost during the

extraction process. This can be correlated with the AA composition analyzed for both protein fractions (Table 3.8), in which ALK-FBPI had a lower SAA content than DES-FBPI.

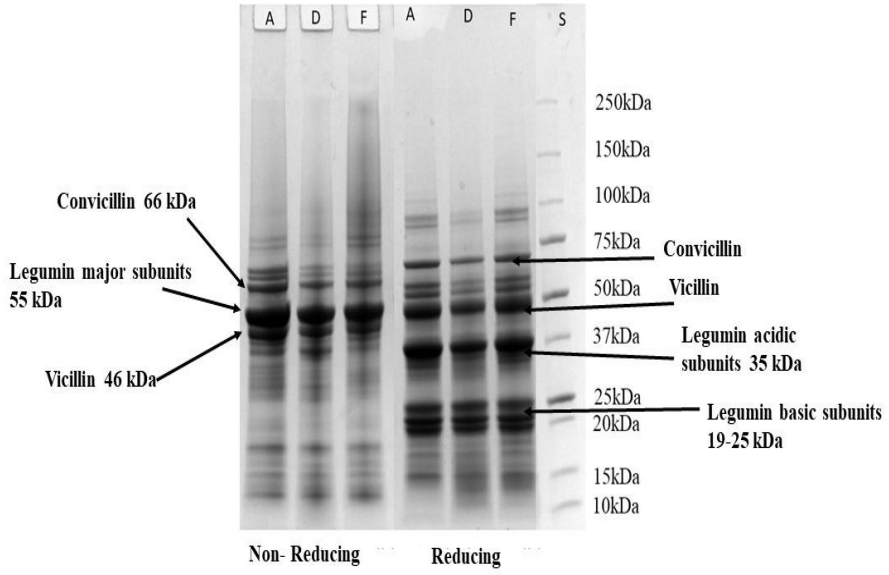


Figure 3.6. SDS-PAGE profile of fava bean flour and extracted protein fractions. Lane A: Alkaline-extracted fava bean protein isolates (ALK-FBPI), lane D: Deep eutectic solvent-extracted fava bean protein isolates (DES-FBPI), lane F: Fava bean flour, and lane S: Molecular weight marker.

3.5. Conclusion

The higher protein content, protein yield, and recovery rate obtained from the DES extraction indicate that it can potentially be scaled up for industrial applications. Further, DES extraction improved the SAA content compared to alkaline-extracted protein isolates. This demonstrates that DES could extract more SAA, thus improving the nutritional value of the protein isolates. Notably, DES extraction increased the amount of the secondary structure component, α-helix, compared to alkaline-extracted proteins. However, the impact on the protein denaturation by the DES extraction was lower, suggesting that it could exhibit better functional properties in

food systems. Overall, DES-based protein extraction, as a green approach, shows a promising technique that could potentially be used as an alternative to the conventional alkaline extraction method. However, further studies on functional performance, potential toxicity, and residual impact are needed before the industrial application of new protein ingredients.

Connection to Chapter 4

Plant protein extraction using DES is still in the early stages of research. The lack of systematic optimization of extraction variables for achieving higher protein yields and protein content, which can compete with conventional alkaline extraction, poses challenges in industrial applications. Therefore, Chapter 3 investigates optimizing the DES system containing choline chloride and glycerol for protein extraction variables, including the choline chloride: glycerol molar ratio, solid: liquid ratio, extraction time, extraction temperature, and water content in the DES. The response surface methodology (RSM) was adopted with the central composite design to choose the optimum extraction conditions to predict protein yield from fava beans. Subsequently, the predicted protein yield was experimentally validated to test the adequacy of the RSM model. With the optimized conditions, a 1:2 w/w choline chloride: glycerol molar ratio, a 1:28 w/w solid: liquid ratio, an extraction time of 1 hrs, an extraction temperature of 50 °C, and a 40% w/w water content in the DES yielded ~65% protein and a protein purity of ~92%. In contrast, the alkaline extraction yielded significantly lower protein (~60%), but similar protein content to the DES extraction. Notably, it was revealed that the denaturation caused by DES extraction was less than that caused by alkaline extraction, as confirmed by analysis of secondary structural components. The promising results obtained from Chapter 3 led to further investigation on the physiochemical, techno-functional properties, and protein quality assessment of DES-extracted fava bean protein isolates, as in Chapter 4. A comparison was made to conventional methods, including alkaline and salt extraction, and commercial soy protein isolates were used as a standard. The protein quality was assessed in terms of amino acid composition and in-vitro protein digestibility. The in-vitro protein digestibility amino acid corrected score was also determined for each protein isolate to understand its nutritional significance, promoting its applicability in food applications with improved protein functionality.

Chapter 4

Effects of novel protein extraction methods on structure-functional properties and protein quality of fava bean protein isolates: A comparative study

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4.1. Abstract

The effects of deep eutectic solvent (DES)-based protein extraction on structural and functional properties of fava bean protein isolate (DESE-FBPI) were determined in comparison to conventional salt (SSE-FBPI) and alkaline (ALKE-FBPI) extraction methods, using commercial soybean protein isolate (CS-PI) as the standard protein. The protein content of DESE-FBPI, ALKE-FBPI, SSE-FBPI, and CS-PI was 92.33 ± 2.28%, 92.50 ± 1.36%, 92.12 ± 1.21%, and 86.97 ± 2.58%, respectively. The solubility of extracted protein isolates was increased in the order of SSE-FBPI > DESE-FBPI > ALKE-FBPI > CS-PI. Differences in the extraction methods reflected a better foaming capacity of DESE-FBPI (132.50±1.66%) at pH 3.0 with 0.5% (w/v) protein concentration when compared to the other protein isolates. At a protein concentration of 0.2% (w/v), the emulsion formed by DESE-FBPI exhibited a higher emulsification index at both pH 3.0 (33.5 ± 0.23 m^2/g) and pH 9.0 (40.83 ± 0.89 m^2/g). Except in DESE-FBPI, FTIR data indicated that all protein isolates had intermolecular β-sheets as protein aggregates, indicating a higher protein denaturation. Moreover, significantly higher *in-vitro* protein digestibility was shown by both ALKE-FBPI and CS-PI as compared to the other protein isolates. CS-PI had a significantly higher *in-vitro*-protein digestibility corrected amino acid score (PDCAAS) of 92.1 ± 0.9% when compared to the other protein isolates. The PDCAAS of DESE-FBPI (76.4 ± 0.5%) was significantly higher than that obtained for ALKE-FBPI (71.1 ± 0.4%) and SSE-FBPI (69.5 ± 0.5%). Different protein isolation methods, therefore, have a considerable impact on protein functionality, structural changes, and protein quality. The overall better functionality of DESE-FBPI demonstrated that DES is a potential alternative agent for plant protein extraction, providing opportunities for increased innovation in plant protein food applications.

4.2. Introduction

Fava bean (*Vicia faba L.*) is an emerging sustainable plant protein source with significant potential to meet increasing global protein demand. It is a versatile legume crop that belongs to the Fabaceae family. It is one of the oldest crops cultivated worldwide, where China, India, Egypt, Ethiopia, Mediterranean countries, Northern Europe, and Northern Africa remain the major producers (Mínguez & Rubiales, 2021; Salvador-Reyes et al., 2023). Nutritionally, the fava bean is a rich source of carbohydrates (47-68%), proteins (25-40%), fiber (11-30%), vitamins, and minerals (Augustin & Cole, 2022; Valente et al., 2018). In addition, it contains a range of health-promoting bioactive compounds such as phenolic compounds, bioactive peptides, gamma-aminobutyric acid (GABA), and L-3,4-dihydroxyphenylalanine (DOPA) (Karkanis et al., 2018; Martineau-Côté et al., 2022). Despite its nutritional value, the presence of antinutritional factors, i.e., tannins, enzyme inhibitors, vicine, and convicine, can diminish the protein digestibility and nutrient bioavailability (Jeganathan et al., 2023; Warsame et al., 2020). In particular, vicine and convicine, which cause favism, are major obstacles to the utilization of fava beans (Khazaei et al., 2019; Pulkkinen et al., 2019). However, the recent development of non-tannin, low vicine, and convicine cultivars and various processing methods has largely benefited in reducing these undesirable compounds (Hou et al., 2018; Khazaei et al., 2019).

The major storage protein of fava bean seeds is salt-soluble globulins, which account for approximately 85% of the total protein (Sharan et al., 2021). The globular protein can be classified into two major subgroups: 11S globulin (legumin) and 7S globulin (vicilin and convicilin) (Ma et al., 2022; Shevkani et al., 2019). The legumin subunit is a hexameric holoprotein, ranging in molecular weight from 300 to 400 kDa, consisting of six polypeptide pairs, each with a molecular weight of approximately 60-80 kDa. Each legumin unit consists of acidic (Legumin α-40 kDa) and

basic (Legumin β-20 kDa) subunits, linked by disulfide bonds (Sharan et al., 2021). In contrast, the 7S fraction is a trimer with an average molecular weight of 150 kDa, lacking disulfide bridging (Liu et al., 2021). This heterogeneity in the fava bean protein composition and structure can affect the final composition of protein isolates, as different extraction methods can selectively extract each component. Commercially, the most common method employed for producing protein isolates is the wet extraction technique, which includes alkaline extraction (AE), isoelectric precipitation (IEP), and salt extraction (SE) (Gençdağ et al., 2021; Sim et al., 2021). Depending on the processing conditions used in the extraction methods, different proportions of protein subunits can be extracted. Therefore, the functional performance of extracted proteins will also be affected. The structure-functional properties of fava bean protein ingredients produced from conventional protein extraction techniques have been well-studied in the literature (Alavi, Chen, Wang, et al., 2021; Krause et al., 2023; Langton et al., 2020; Vogelsang-O'Dwyer et al., 2020). However, extensive protein denaturation and aggregation that occur during conventional methods often negatively impact the techno-functional properties, thus reducing their applicability in food systems (Boukid & Castellari, 2022; Eckert et al., 2019). Therefore, an alternative novel extraction method is needed in the plant protein industry to produce functionally superior protein isolates. Hence, the deep eutectic solvent (DES) has recently become a potential candidate for protein extraction owing to its widely recognized sustainability characteristics such as biodegradability, low toxicity, recyclability, easy preparation, and low cost (Chen & Mu, 2019; Hansen et al., 2021; Hewage et al., 2022; Hewage et al., 2024). The unique characteristic of the intermolecular hydrogen bond network formed between the hydrogen bond donor and acceptor in DES is believed to be involved in its special solubilizing and stabilizing effect on proteins during protein extraction (El Achkar et al., 2021; Liu et al., 2018). Thus, the interaction between dispersed protein and DES

(specific hydrogen and electrostatic bonds) may alter the protein's structure and dynamic behavior (Sanchez-Fernandez & Jackson, 2021). Furthermore, the fundamental knowledge of DES-induced structural changes is still in its early research stage, and its impact on functional properties remains to be addressed.

A recent study reported that a DES system containing choline chloride (ChCl)/glycerol (Gly) could lead to the irreversible denaturation of soy proteins (Chen et al., 2021). In another study (Yue et al., 2021), butanediol isomers-based DES extraction reported an increased amount of α-helix and changes in solubility, foaming properties, and thermal stability of oat proteins. Our recent study (Hewage et al., 2024) demonstrated that ChCl/Gly DES system could extract protein isolates with high protein content (>90%) from fava beans with a lower degree of denaturation compared to AE. However, the current literature on the functional properties of DES-extracted proteins in comparison to other extraction techniques is significantly lacking. We hypothesize that the ChCl/Gly DES system enhances the functionality of fava bean protein isolates over conventional extraction methods. Therefore, the main objective of this work was to investigate the structure and functional properties of fava bean protein isolates obtained through DES extraction, in comparison to those obtained from conventional methods, AE and SE, and commercially produced soybean protein isolates. The data generated from this study will contribute novel insights to a fundamental understanding of the potential behavior of DES-extracted proteins in different food systems, providing a basis for further studies. This will enable industries to create considerable opportunities for innovation in the plant protein industry across the value chain, thereby increasing the utilization of fava beans for new food applications.

4.3. Materials and Methodology

4.3.1. Materials and chemicals

Dehulled fava bean seeds (Snowbird variety) were purchased from Prairie Fava (Glenboro, MB, Canada). Laemmli sample buffer, 2-mercaptoethanol, Precision Plus protein standard (10-250 kDa), Coomassie Blue R-250, and 4-15% Mini-PROTEAN® TGX™ precast protein gels were purchased from Bio-Rad Laboratories Ltd. (Mississauga, ON, Canada). Pancreatic trypsin type IX-S (13,000–20,000 Na-benzoyl-L-arginine ethyl ester (BAEE) units/mg protein, T0303), α-chymotrypsin (bovine pancreatic chymotrypsin type II, ≥ 40 N-Benzoyl-L- Tyrosine Ethyl Ester (BTEE) units/mg protein, C4129), and peptidase (protease from *Streptomyces griseus* Type XIV, ≥ 3.5 units protease/mg solid, P5147), were purchased from Sigma-Aldrich. St. Louis, MO, USA. Commercial soybean protein isolates (CS-PI) and all other analytical grade chemicals were purchased from Fisher Scientific (Ottawa, ON, Canada).

4.3.2. Fava bean seed processing

Fava bean seeds were processed according to the method described in our previous study (Hewage et al., 2024). The average particle size D (0.5) of the milled flour was 41.14 ± 1.72 µm.

4.3.3. Methods

In this study, three different protein extraction methods were employed: ChCl/Gly-based DES extraction (DES), alkaline extraction (AE) followed by isoelectric precipitation (IEP), and salt extraction (SE). All extractions were performed in triplicate. CS-PI was used as a standard for comparison with the fava bean protein isolates.

4.3.4. Protein extraction

4.3.4.1. DES extraction

DES protein extraction was performed using optimized conditions developed in our previous study (Hewage et al., 2024). First, fava bean flour (FBF) was mixed with 60% DES containing ChCl and Gly (1:2 molar ratio, w/w) and 40% water (w/w) at a 28:1 liquid: solid ratio. It was then homogenized at a speed of 20,000 rpm for 2 min (Model 850, Fisher Scientific, Ottawa, ON, Canada), followed by protein extraction using a circulating water bath (Model 6200 H7, Fisher Scientific Inc, Pittsburgh, PA, USA) at 200 rpm, 50 °C for 1 hrs. The supernatant collected after centrifugation (1507 g, 10 min, 4 °C) was dialyzed (3.5 kDa molecular weight cut-off, MWCO) at 4 °C for 5 days, with continuous refreshment with distilled water. Finally, the resulting protein solution was freeze-dried and stored at -20 °C as the deep eutectic solvent extracted fava bean protein isolate (DESE-FBPI).

4.3.4.2. Alkaline extraction (AE)

Alkaline extraction followed by isoelectric precipitation was performed based on the method described previously (Karaca et al., 2011) with slight modifications. FBF was dispersed in a 1:10 (w/v) solid-to-liquid ratio and homogenized (Model 850, Fisher Scientific, Ottawa, ON, Canada) at 14,000 rpm for 2 min. The slurry was then adjusted to pH 9.5 using 5 M NaOH. After 2 hrs of stirring (200 rpm) at 22 ºC, the mixture was centrifuged at 17,709 g, 4 ºC for 20 min. The supernatant was adjusted to pH 4.5 using 1 M HCl to precipitate the proteins. Thereafter, precipitated proteins were collected using centrifugation, and protein pellets were dispersed in distilled water. Before freeze-drying, the protein slurry was adjusted to pH 7.0 using 1 M NaOH, and the dried protein was stored at -20 °C as the alkaline extracted fava bean protein isolate (ALKE-FBPI).

4.3.4.3. Salt extraction (SE)

Salt-extracted fava bean protein isolates were obtained according to the protocol published previously (Karaca et al., 2011) with slight modifications. FBF was mixed with a 5% (w/v) aqueous solution of sodium sulfate at a 1:10 (w/v) ratio and homogenized (Model 850, Fisher Scientific, Ottawa, ON, Canada) at 14,000 rpm for 2 min. The slurry was then adjusted to a pH of 7.0 with 0.1 M NaOH and stirred for 2 hrs at 200 rpm, and 22 °C. The mixture was centrifuged (17,709 g, 20 min, 4 °C), and the supernatant dialyzed using 3.5 kDa MWCO tubing at 4 °C for 72 hrs by changing the water daily, freeze-dried, and stored at -20 °C as the salt-extracted fava bean protein isolate (SSE-FBPI).

4.3.4.4. Protein yield and protein recovery rate

Protein yield (PY) and protein recovery rate (PR) of extracted proteins were calculated using equations (1) and (2), respectively.

$$PY\%\,(DW) = Ep \times P/Rp \times 100 \quad (1)$$

$$PR\,\%\,(DW) = (Ep \times P)/Rw \times 100 \quad (2)$$

Where *Ep* is the Weight (g) of extracted protein on a dry weight (DW) basis, *P* is the percentage of purity of extracted protein in DW, *Rp* is Protein content (g) in initial raw material on a DW basis, *Rw* is the weight (g) of initial raw material in DW basis.

4.3.5. Proximate analysis

Extracted protein fractions and the CS-PI were analyzed for moisture, crude protein, ash, and crude fat according to the AOAC method (AOAC, 2000). Total nitrogen was converted to crude protein content using a single factor of 6.25 to enable direct comparison between all protein isolates studied. The total carbohydrates (CHO) were estimated using the difference method.

4.3.6. Physiochemical characterization

4.3.6.1. Surface hydrophobicity (S_0)

The surface hydrophobicity of the protein was determined using 1-anilino-8-naphthalene sulfonate (ANS) as a fluorescent probe, according to the method explained by Osemwota et al. (2021). The stock solution was prepared at a concentration of 10 mg/mL by mixing the protein fraction with 0.1 M phosphate buffer (pH 7.0) for 2 hrs, followed by centrifugation at 10,000 g for 10 min. The supernatants were then diluted to final concentrations of 50-250 μg/mL. A 20 μL aliquot of a 0.8 M ANS solution, prepared in the same buffer, was added to the above series of protein concentrations. The fluorescence intensity (FI) of ANS-conjugates was measured using a spectrofluorometer (Jasco-FP-6300, Tokyo, Japan) at excitation and emission wavelengths of 390 nm and 470 nm, respectively. The FI versus protein isolate concentration was plotted, and the slope (S_0) was calculated by linear regression and used as an index of the surface hydrophobicity of the proteins.

4.3.6.2. Particle size distribution

The particle size distribution of protein fractions (dry samples) was determined using a static laser light diffraction method (Mastersizer 3000, Malvern Instruments Ltd, Malvern, UK). Before analysis, the samples were sieved through a 1 mm mesh and introduced into the dispersing unit at a feeding rate of 20%. The refractive index and absorption index of the protein samples were set at 1.45 and 0.1, respectively, until a laser obscuration of 3-4% was achieved. The results were reported as the volume-weighted mean particle diameter (D 4,3), surface-area-weighted mean particle diameter (D 3,2), and volume percentiles at the 10th [D (0.1)], 50th [D (0.5)], and 90th [D (0.9)] percentiles.

4.3.6.3. Attenuated total reflectance Fourier-transformed infrared spectroscopy (ATR-FTIR)

The changes in the secondary structure of extracted proteins and CS-PI were characterized using an INVENIO S (Bruker Scientific LLC., MA, USA) ATR-FTIR. Infrared (IR) spectra generated in the range of 400-4000 cm^{-1} were collected (resolution 4 cm^{-1}, 120 scans), and the curve fitting procedure was performed to locate the amide I peak (1600-1700 cm^{-1}) after the normalization of the spectral data using Origin 2021 software (Origin lab cooperation, MA, USA). Then, the second derivative analysis was carried out with baseline reduction and a Savitzky-Golay smooth function with a window size of 9, followed by a multiple peak fitting procedure to quantify the secondary structure components under the Gaussian function, using the same software.

4.3.6.4. Differential scanning spectroscopy (DSC)

Changes in the thermal properties of protein fractions were characterized using differential scanning calorimetry (DSC) (Model DSC2500, TA Instruments- Waters LLC, DE, USA). Before analysis, sample moisture was removed by placing the samples in P_2O_5, contained in a hermetically sealed desiccator, for 7 days. Approximately 3-5 mg of dry sample was weighed into T-Zero hematic aluminum pans and sealed hermetically with lids. Samples were heated from 30 to 250 ºC at a ramping rate of 10 ºC/min under a nitrogen purging flow of 50 mL/min, after the sample was equilibrated at 30 ºC. Heat flow was measured against a reference pan, and after collecting thermodynamic data, TA Universal Analysis 2000 software (Model DSC2500, TA Instruments-Waters LLC, DE, USA) was used to analyze the data**.**

4.3.6.5. Sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE)

Gel electrophoresis was performed under both reducing and non-reducing conditions using a 4-15% Mini-Protean TGX precast gel in a Mini-Protean II electrophoresis unit (Bio-Rad Laboratories Inc., CA, USA), according to the method outlined by Raikos et al. (2014) with

modifications. Protein samples (4 mg/mL) were mixed with an equal volume of Laemmli sample buffer, either with or without 2-mercaptoethanol (reducing or non-reducing conditions, respectively). Samples were then heated at 90 °C for 5 min and allowed to cool to room temperature. 10 µL of Precision Plus protein standard (Bio-Rad Laboratories Inc., CA, USA) and 5 µL of each sample were loaded into precast gels. After running the electrophoresis at a constant rate of 150 V, gels were stained with Coomassie Brilliant Blue R-250 for 1 hrs, followed by destaining for 2 hrs.

4.3.7. Functional properties

4.3.7.1. Protein solubility (PS)

PS at different pH (2.0-12.0) was determined according to the method adopted by Barac et al. (2010) with slight modifications. Approximately 0.1 g of protein isolate was dissolved in 20 mL of Milli-Q water and stirred at 22 °C and 200 rpm for 1.5 hrs to obtain a uniform dispersion. The suspensions were then adjusted with 1 M NaOH or 1 M HCl to pH 3.0-12.0. After that, the solution was stirred using the same conditions as above and centrifuged (15,000 g, 15 min, 22 °C). The soluble protein content in the supernatant was determined using the Kjeldahl method (AOAC, 2000). The protein solubility was expressed as the amount of soluble protein in the supernatant per gram of total protein in the initial sample.

4.3.7.2. Water and oil holding capacity (WHC and OHC)

WHC and OHC were determined using the method described by Malomo et al. (2014) with some modifications. For WHC, 1 g of protein sample was dispersed in 20 mL phosphate buffer (pH 7.0) in a pre-weighted Falcon tube. The mixture was vortexed for 2 min, allowed to stand for 30 min, and then centrifuged at 4000 g for 30 min at 22 °C. The supernatant was decanted, and the tubes were inverted to drain the excess water for 15 min. The residue weight was then recorded.

OHC was measured by dispersing the protein sample (1 g) in 10 mL of canola oil using the same procedure as WHC. WHC and OHC were calculated using equation (3).

$$WHC\ or\ OHC\% = \frac{Weight\ of\ the\ water\ or\ oil\ absorbed\ by\ sample}{Weight\ of\ the\ initial\ sample} \times 100 \quad (3)$$

4.3.7.3. Emulsifying activity index (EAI) and emulsion stability index (ESI)

EAI and ESI were determined using the procedure described by Karaca et al. (2011) with modifications. Ten ml of 0.1, 0.5, and 1% (w/v) protein solutions were prepared at pH 3.0, 5.0, 7.0, and 9.0 using 0.1 M acetate (pH 3.0 and 5.0), phosphate (pH 7.0), and Tris-HCl (pH 9.0) buffers respectively, followed by addition of 5 mL canola oil. The oil/water mixture was homogenized at 20,000 rpm for 1 min (Model 850, Fisher Scientific, Ottawa, ON, Canada). A 50 µL emulsion sample was immediately taken from the bottom of the tube and diluted in 5 mL of 0.1% (w/v) sodium dodecyl sulfate (SDS) solution. The resulting suspension was vortexed for 10 sec, and the absorbance at 500 nm was measured immediately and after 10 min using a UV-spectrophotometer (Genesys 150, Madison, USA). The EAI and EAS were calculated using equations (4) and (5), respectively.

$$EAI\ (m^2/g) = \frac{2 \times 2.303 \times A_0 \times N}{c \times \varphi \times 10000} \quad (4)$$

$$ESI\ (\text{min}) = \frac{A_0}{\Delta A} \times t \quad (5)$$

Where A_0 is the absorbance of the diluted emulsion immediately after homogenization, N is the dilution factor (×100), c is the weight of protein per volume (g/mL), φ is the oil volume fraction

of the emulsion, ΔA is the change in absorbance between 0 and 10 min (A_0–A_{10}), and t is the time interval, 10 min.

4.3.7.4. Foaming capacity (FC) and foaming stability (FS)

FC and FS were estimated based on the method described by Vogelsang-O'Dwyer et al. (2020) with slight modifications. Twenty mL of 0.1, 0.5, and 1% (w/v) protein solutions were prepared in a 50 mL measuring cylinder, using 0.1 M acetate (pH 3.0 and 5.0), phosphate (pH 7.0), and Tris-HCl (pH 9.0) buffers. The protein solution was homogenized at 11,000 rpm for 2 min (Model 850, Fisher Scientific, Ottawa, ON, Canada) using a 20 mm shaft to generate foams. After 10 sec, the initial foam volume was recorded (V_0), allowed to stand for 30 min at room temperature (22 °C), and again, foam volume was measured (V_1). FC and FS were calculated using the following equations (6) and (7).

$$FC\% = \left(\frac{V_0}{20mL}\right) \times 100 \quad (6)$$

$$FS\% = \left(\frac{V_1}{V_0}\right) \times 100 \quad (7)$$

4.3.7.5. Least gel concentration (LGC)

LGC of protein fractions was determined using the method adopted by Vogelsang-O'Dwyer et al. (2020) with modifications. Proteins were dispersed in 0.1 M acetate (pH 3.0 and 5.0), phosphate (pH 7.0), and Tris-HCl (pH 9.0) buffers at concentrations ranging from 5% to 20% (based on protein weight w/v) and hydrated at 4 °C for 16 hrs. Prepared samples were heated at 90 °C for 1 hrs, cooled immediately using running water, and stored at 4 °C overnight. The LGC was defined as the concentration at which the gel did not slip out when the tubes were inverted.

The gels formed were categorized as firm (F) when the gel could be removed from the tube without breaking, whereas soft gels (S) formed when they fell apart.

4.3.8. Protein quality assessment

4.3.8.1. Amino acid (AA) composition

AA contents of protein isolates were determined using the AOAC Official Method 982.30. First, 24 hrs acid (HCl, 6 N) hydrolysis was carried out to estimate most amino acids (AOAC International, 2012), except methionine, cysteine, and tryptophan. For the determination of methionine and cysteine, protein samples were first oxidized with performic acid (AOAC Official Method 985.28), followed by hydrolysis, as described above. For tryptophan analysis, alkaline hydrolysis was performed using ISO protocol 13904:2005 (ISO, 2005). All hydrolysed amino acids were then derivatized and separated using a Shimadzu ultra-high performance liquid chromatography (UPLC) system (Kyoto, Japan) equipped with a Waters AccQ C18 column (100 mm × 2.1 mm, 1.7 µm). AAs were quantified based on their hydrated molecular weights, and the data were expressed as a percentage by weight. As a reference, the NIST soy flour standard (Reference Material 3234) was used for quality control purposes.

4.3.8.2. Amino acid score (AAS)

The AA composition was used to estimate AAS with preschool children's scoring patterns (2 to 5 years old) as a reference (FAO/WHO, 1991). AAS was calculated using equation 8, and the essential AA with the lowest AAS was reported as the first limiting AA.

$$\text{AAS} = \frac{\text{mg of amino acid in test protein}}{\text{mg of amino acid in requirement pattern}} \times 100 \quad (8)$$

4.3.8.3. *In vitro* protein digestibility (IVPD) and *in vitro*-protein digestibility corrected amino acid score (IV–PDCAAS)

IVPD of protein isolates was determined using the pH-drop method outlined by Hsu et al. (1977), with minor modifications (Tinus et al., 2012) as described by Sá et al. (2023). Briefly, protein suspension prepared in distilled water (6.25 mg/mL) was adjusted to pH 8.0 using 0.1 M HCl or 0.1 M NaOH at 37 °C. A 1 mL aliquot of an enzymatic mixture containing 1.6 mg/mL of trypsin porcine, 3.1 mg/mL of α-chymotrypsin, and 1.3 mg/mL of peptidase, maintained in an ice bath at pH 8.0, was added to the protein solution at a ratio of 1:10 v/v under continuous stirring conditions at 37 °C. After 10 min of proteolytic enzyme reaction, the pH of the sample was measured. IVPD was estimated as a percentage of digestible protein based on the pH variation after 10 min ($\triangle pH_{10\ min}$), as shown in equation (9). The IV-PDCAAS was then calculated by multiplying the estimated IVPD value by the lowest AAS (Section 4.3.8.2), as obtained based on the scoring pattern of preschool children (2 to 5 years old) (FAO/WHO, 1991), as shown in equation (10).

$$IVPD\ (\%) = 65.66 + 18.10\ \times \triangle\ pH_{10min} \quad (9)$$

$$IV\text{–}PDCAAS\ (\%) = IVPD\ \times AAS \quad (10)$$

4.4. Statistical analysis

Protein extractions were performed in triplicate, and the resulting protein isolates were combined for each extraction method. All analyses of protein fractions were performed in triplicate, and the results are expressed as the mean ± standard deviation (SD). Statistical analysis was performed using one-way and two-way analysis of variance (ANOVA) followed by a Tukey test, by MINITAB statistical software (MINITAB version 20, LLC, PA, USA), with a significance level of $P < 0.05$.

4.5. Results and Discussion

4.5.1. Protein extraction yield and recovery rate

Table 4. 1. Protein extraction yield and recovery rate of deep eutectic solvent, alkaline, and salt-extracted fava bean protein isolates

Protein isolate	Protein yield %	Recovery rate %	Protein content %
DESE-FBPI	65.42 ± 6.53^{a}	23.15 ± 2.31^{a}	92.14 ± 2.11^{a}
ALKE-FBPI	60.77 ± 1.17^{ab}	21.74 ± 0.19^{a}	92.71 ± 1.33^{a}
SSE-FBPI	56.06 ± 3.26^{b}	17.90 ± 1.00^{b}	92.12 ± 1.62^{a}

All data were analyzed using variance analysis (ANOVA) followed by Tukey's test (n=3) and expressed on a dry weight basis. Different superscripts within a column indicate a significant difference ($P < 0.05$). DESE-FBPI, deep eutectic solvent-extracted fava bean protein isolate; ALKE-FBPI, Alkaline-extracted fava bean protein isolate; SSE-FBPI, Salt-extracted fava bean protein isolate.

Protein yield, protein content, and recovery rate of DESE-FBPI, ALKE-FBPI, and SSE-FBPI proteins are presented in Table 4.1. Protein yield significantly differed among the various extraction methods studied, and analysis of variance revealed that the type of extraction method was a significant factor ($P < 0.05$). DESE-FBPI yielded the highest protein content, followed by ALKE-FBPI and SSE-FBPI, but with no significant difference in protein content ($P < 0.05$). The protein recovery rates of DESE-FBPI and ALKE-FBPI were not significantly different from each other, but they were significantly higher than those of SSE-FBPI. Similar to our study, a significantly higher protein yield was observed for tender bamboo shoots when extracted with DES (39.16 mg/g) compared to AE (23.88 mg/g). However, previously reported values for protein yield

and protein content for oats (Yue et al., 2021), rapeseed, and evening primrose cake (Grudniewska et al., 2018) for DES extraction were much lower than the results obtained in this study. The differences in raw materials, DES components, and extraction conditions may have contributed to the low extractability of the previously reported raw materials. The AE result is comparable to Langton et al. (2020), who reported a protein extraction yield of 61.7% from fava beans. Abdel-Aal et al. (1986) reported lower protein recovery for chickpeas when using AE compared to micellar precipitation. Stone et al. (2014) demonstrated that SE and AE could produce an average protein yield of 68.2-74.8% and 62.6-76.7%, respectively, from pea proteins. The study by Karaca et al. (2011) demonstrated that protein isolates produced from fava beans using the AE method had an 84.14% protein content, which is higher than the 81.98% obtained with the SE method. A similar trend was also observed with pea proteins, as reported by Stone et al. (2014) and Tanger et al. (2020). DES efficiently extracted proteins with high content, likely due to unique hydrogen bond interactions that drive protein dissolution and stability (Liu et al., 2018).

4.5.2. Proximate composition

Table 4. 2. Proximate composition of protein isolates produced by different extraction methods

Protein Ingredients	Moisture %	Ash %	Fat %	Protein %	Total CHO*%
DESE- FBPI	7.75 ± 0.97^{a}	3.19 ± 0.23^{c}	1.87 ± 0.97^{ab}	92.14 ± 3.14^{a}	2.8
ALKE- FBPI	1.37 ± 0.64^{c}	3.18 ± 0.08^{c}	3.45 ± 0.49^{a}	92.71 ± 1.33^{a}	0.66
SSE- FBPI	4.18 ± 0.19^{b}	4.75 ± 0.17^{a}	1.24 ± 0.42^{b}	92.12 ± 1.62^{a}	1.89
CS-PI	5.37 ± 0.29^{b}	4.17 ± 0.05^{b}	0.98 ± 0.64^{b}	86.97 ± 3.42^{a}	7.88

DESE-FBPI (Deep eutectic solvent-extracted fava bean protein isolate), ALKE-FBPI (Alkaline-extracted fava bean protein isolate), SSE-FBPI (Salt-extracted fava bean protein isolate), and CS-

PI (Commercial soybean protein isolate). Results are presented as the mean ± standard deviation (n =3) on a dry weight basis. Different superscripts within a column indicate significant differences ($P < 0.05$). * Calculated by differential from 100%.

The proximate composition of all extracted protein isolates is shown in Table 4.2. All protein isolates, except for CS-PI, had a protein content of ~92%, with no significant differences ($P > 0.05$). Protein levels found in ALKE-FBPI and SSE-FBPI are comparable to those reported in previous studies (Jeganathan et al., 2023; Vioque et al., 2012; Vogelsang-O'Dwyer et al., 2020). In contrast, Karaca et al. (2011) reported that 84.14% and 81.98% of protein levels were obtained from fava beans using SE and AE methods, which are lower than those produced in our study. The lipid content of all protein isolates was below 2% except in ALKE-FBPI, consistent with previously reported data (Dhull et al., 2021). Protein isolation with AE, followed by IEP, may have increased the interaction between lipids and proteins. Lower fat content in DESE-FBPI may benefit the solubility properties of food applications. The total ash content of isolates ranged from 3-5%, among which SSE-FBPI had a significantly higher amount, followed by CS-PI, whereas ALKE-FBPI and DESE-FBPI had similar levels. The high ash content of isolates may increase the mineral composition when combined with a diet poor in minerals. However, ash content of more than 1% in isolates can negatively impact bakery food applications due to the dilution effect of proteins (Cauvain & Young, 2007). The high ash content in protein isolates may be due to residual salts used in the extraction process, and the salts generated through isoelectric precipitation might not have been completely removed during the washing steps. The total carbohydrate (CHO) content of the extracted isolates was very low, ranging from 0.7% to 3%, except in the commercial soy proteins. This indicates that DES extraction efficiently excludes the CHO components, similar to conventional methods, thus improving the protein content.

4.5.3. Physiochemical characterization

4.5.3.1. Surface hydrophobicity (S_o)

Surface hydrophobicity (S_o) can be used as an indicator to provide information about protein conformation and its stability (Jiang et al., 2015), and it has a significant correlation with surface-related functional properties (Kato & Nakai, 1980). As shown in Table 4.3, the S_o values obtained for all isolation methods were significantly different (P<0.05). The highest S_o was observed for CS-PI and decreased in order of ALKE-FBPI > SSE-FBPI > DESE-FBPI. This implies the presence of more hydrophobic clusters in CS-PI than in fava bean protein isolates. Commercially produced protein isolates often undergo a certain degree of denaturation due to the harsh conditions employed and the additional actions of the industrial spray-drying process (He et al., 2015). As a result, buried hydrophobic amino acids are exposed, leading to an irreversible change in protein conformation (Wang et al., 2014), which can negatively impact protein functionality. S_o of protein isolates prepared from AE was three times lower than those of CS-PI, although it was still extracted under higher pH conditions similar to those used in commercial extraction. This can be attributed to the fact that spray drying employed in the industrial process causes more protein denaturation than the freeze-drying method. Compared to AE and SE, the protein isolates from DES extraction had significantly lower S_0, indicating SE also possibly influenced the degree of denaturation by disrupting the non-covalent electrostatic bonds. Protein extracted by salt and the DES is typically composed of a mixture of globulins and albumins (Hewage et al., 2024; Liu et al., 2008), whereas AE followed by IEP results in fractionation, as the soluble albumin fraction is mostly lost during the isolation process (Yang et al., 2022). The surface hydrophobicity of globulin is generally reported to be higher than that of albumin (Malomo et al., 2014). Therefore, isolates produced by AE may exhibit relatively higher So values than those produced by DESE-FBPI and SSE-FBPI. The

differences in S_0 may be due to variations in protein content, amino acid (AA) composition, and surface charge distribution, which are influenced by different extraction methods.

Table 4. 3. Surface hydrophobicity and thermal stability of protein isolates obtained using various extraction methods.

Protein Ingredients	SH (S_o)	Thermal properties		
		T_o (ºC)	T_d (ºC)	ΔH (J/g)
DESE-FBPI	1.21 ± 0.22^{c}	92.04 ± 9.53^{a}	126.10 ± 3.55^{a}	116.97 ± 9.76^{a}
ALKE-FBPI	3.10 ± 0.51^{b}	75.70 ± 4.20^{a}	131.66 ± 5.49^{a}	60.35 ± 3.02^{b}
SSE-FBPI	2.20 ± 1.02^{bc}	96.12 ± 6.05^{a}	135.29 ± 6.84^{a}	117.39 ± 9.57^{a}
CS-PI	9.42 ± 0.77^{a}	90.31 ± 6.92^{a}	138.41 ± 3.50^{a}	71.74 ± 4.63^{b}

Surface hydrophobicity (S_0) and thermal properties; T_o – onset temperature, T_d- peak transition temperature, ΔH- enthalpy, of DESE- FBPI (Deep eutectic solvent-extracted fava bean protein isolate), ALKE-FBPI (Alkaline-extracted fava bean protein isolate), SSE-FBPI (Salt-extracted fava bean protein isolate), and CS-PI (Commercial soybean protein isolate). All data were analyzed by analysis of variance followed by Tukey's range test for mean separation (n=3). Different superscripts within a column indicate a significant difference ($P< 0.05$).

4.5.3.2. Particle size distribution

The effect of extraction methods on the particle size distribution and average particle size of all the protein isolates is shown in Figure 4.1 and Table 4.4**,** respectively**.** The volume-weighted mean diameter (D 4,3) reflects the size of the coarse particulates that make up the bulk of the sample, and it is sensitive to larger particle size distributions. Meanwhile, surface-area mean diameter (D 3,2) is most sensitive to the fine particulates in the size distribution. The particle size

distribution of all extracted protein isolates, including CS-PI, exhibited a monomodal distribution in the range of ~ 0.5-1000 μm (Figure 4.1). The particle size distribution varied significantly ($P<0.05$) depending on the extraction method. ALKE-FBPI was significantly distributed in the larger particle size range (~5-1000 μm) compared to all the other isolates across all parameters investigated. This may be due to acid precipitation, which could induce aggregation, resulting in larger and coarser particles. As shown in Table 4.4, DESE-FBPI generally had a significantly lower size distribution than most other protein isolates. The higher solubility of proteins during DES extraction, as demonstrated by the higher protein yield (Table 4.1), may be responsible for producing finer-sized particles. For D (3,2) values, no significant difference was found between SSE-FBPI and DESE-FBPI. However, SSE-FBPI exhibited a significantly lower distribution for the 10^{th} volume percentile [D (0.1)], followed by DESE-FBPI, CS-PI, and ALKE-FBPI. In both DES and SE processes, subsequent protein recovery was achieved by using the dialysis method. Thus, the membrane's pore size determines the molecular size of proteins that can pass through. This may have affected the lower particle size distribution of both SSE-FBPI and DESE-FBPI. In the case of [D (0.5)], except for ALKE-FBPI, the other protein isolates were distributed in the ~44-51 μm range ($P > 0.05$), indicating that 50% of the sample consisted of particles within this size range. The size range observed for ALKE-FBPI in this study is relatively higher when compared to values reported by Vogelsang-O'Dwyer et al. (2020). This may be due to the protein aggregation under the high pH conditions in alkaline extraction. Furthermore, John & Mansuri (2018) reported higher D (3,2) and D (4,3) values for CS-PI compared to this study. Differences may have been accounted for due to variations in the extraction process and methodology adopted for sample preparation for laser diffraction.

Table 4. 4. Particle size distribution (µm) of extracted protein isolates by different methods

Parameter (µm)	DESE-FBPI	ALKE-FBPI	SSE-FBPI	CS-PI
D (0.1)	11.00 ± 0.25^{c}	26.20 ± 0.85^{a}	8.26 ± 0.01^{d}	14.85 ± 0.07^{b}
D (0.5)	43.50 ± 0.21^{b}	150.00 ± 4.24^{a}	49.40 ± 0.00^{b}	50.70 ± 0.57^{b}
D (0.9)	118.00 ± 1.41^{d}	375.00 ± 4.24^{a}	190.00 ± 0.00^{b}	149.50 ± 2.12^{c}
D (4,3)	56.25 ± 1.34^{d}	178.50 ± 3.54^{a}	81.30 ± 0.57^{b}	69.10 ± 1.13^{c}
D (3,2)	19.60 ± 0.28^{c}	52.00 ± 1.84^{a}	16.15 ± 0.07^{c}	30.35 ± 0.21^{b}

D [4,3]: Volume weighted mean particle diameter, D [3,2]: Surface area-weighted mean particle diameter, D (0.1): 10^{th} volume percentile, D (0.5): 50^{th} volume percentile, D (0.9): 90^{th} volume percentile of DESE-FBPI (Deep eutectic solvent-extracted fava bean protein isolate), ALKE-FBPI (Alkaline-extracted fava bean protein isolate), SSE- FBPI (Salt-extracted fava bean protein isolate), and CS-PI (Commercial soybean protein isolate). All data were analyzed by analysis of variance followed by Tukey's range test (n=3). Different superscripts within a row indicate a significant difference ($P< 0.05$).

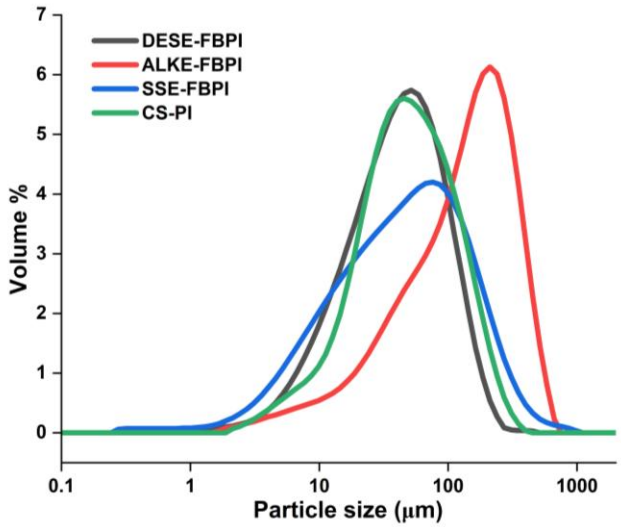

Figure 4. 1. Particle size distribution of extracted protein isolates. DES-FBPI, Deep eutectic solvent-extracted fava bean protein isolate; ALKE-FBPI, Alkaline-extracted fava bean protein isolate; SSE-FBPI (Salt-extracted fava bean protein isolate) and CS-PI (Commercial soybean protein isolate).

4.5.3.3. Changes in protein secondary structures due to extraction methods

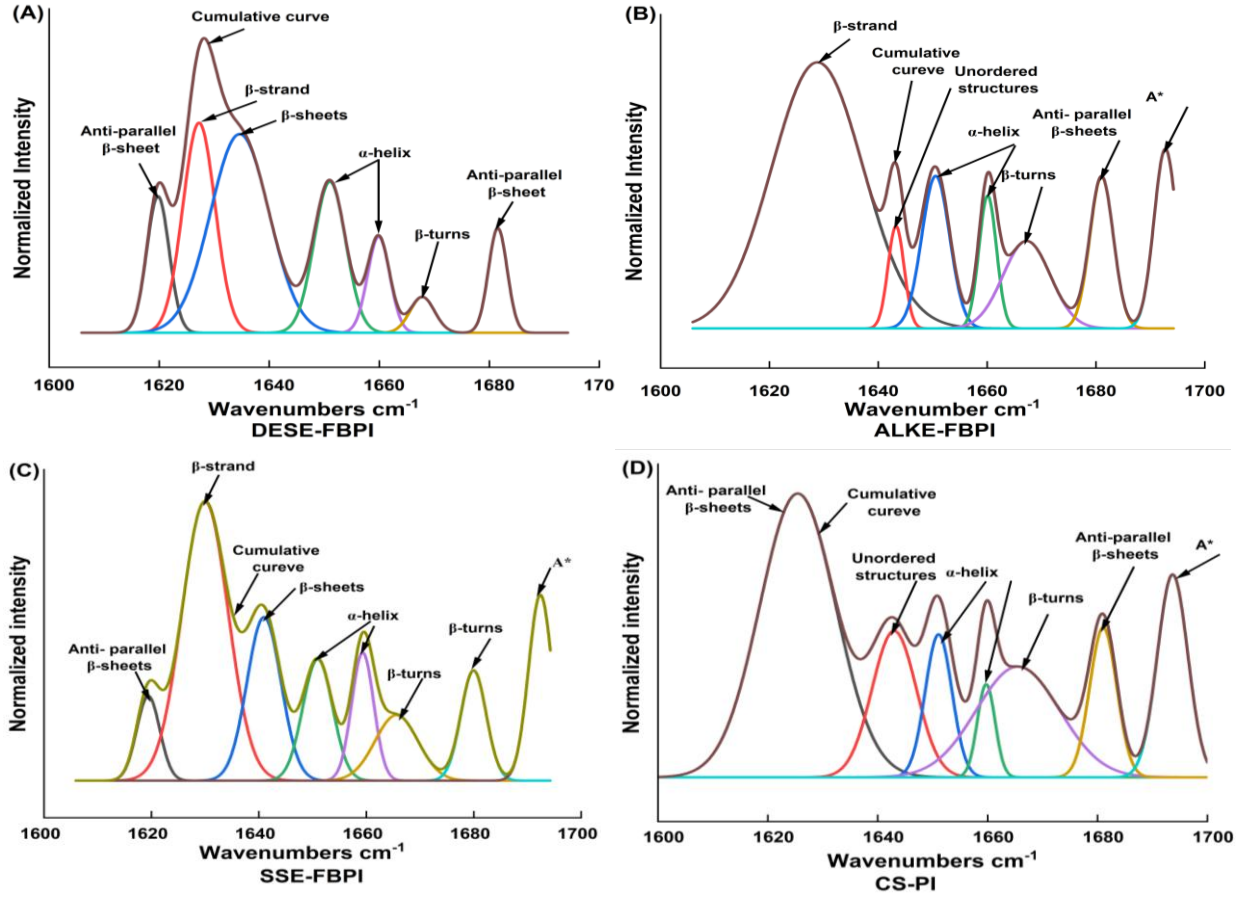

Figure 4.2. (A-D) Peak fitting of amide I with the relative proportion of each secondary structure of (A) Deep eutectic solvent-extracted fava bean protein isolate (DESE-FBPI), (B) Alkaline-extracted fava bean protein isolate (ALKE-FBPI), (C) Salt-extracted fava bean protein isolate (SSE-FBPI), and (D) Commercial soybean protein isolate (CS-PI). A*-Intermolecular β-sheet as aggregates.

FTIR provides qualitative and quantitative estimates of protein secondary structure components, allowing for the evaluation of structural changes in proteins during various processing methods. Changes in the secondary structures of all protein isolates extracted by different methods are presented in Figure 4.2. The amide I peak derived from each protein from the FTIR spectra was analyzed for peak fittings of secondary structure components, and their relative proportion of each secondary structure component was quantified as spectral weights (Table 4.5). Based on the results, secondary structure elements varied depending on the extraction method. The major secondary structure component for all four protein samples was β-structures. In General, legume proteins comprise relatively higher β-structures than the α-helix content (Shevkani et al., 2019), and our results are consistent with those reported for proteins extracted by AE from fava beans (Martínez-Velasco et al., 2018; Nivala et al., 2021). The band intensity between 1619-1640 and 1681, 1650-1660, 1665-1679, and 1692-1693 and 1642-1643 cm^{-1} is associated with β-structures (anti-parallel β-sheets, β-strands and β-sheets), α-helix, β-turns, intermolecular β-sheets as aggregates, and unordered structures (Shevkani et al., 2019). The β-structures content of all protein isolates ranged between 33-40%; CS-PI accounted for the highest amount (39.99%), followed by SSE-FBPI (37.53%), ALKE-FBPI (35.07%) and DESE-FBPI (33.42%). The lower amount of β-structures in DESE-FBPI is comparable to the results reported by Lin et al.(2022) for DES-extracted seabuckthorn seed meal proteins. A decrease in the relative proportion of β-sheets may increase protein flexibility (Carbonaro et al., 2012), showing improved functional performance, as evidenced by the DESE-FBPI. Moreover, ALKE-FBPI and CS-PI possessed several peaks centered at 1692 and 1643 cm^{-1} and 1693-1642 cm^{-1}, which were assigned to be intermolecular β-sheet aggregates and unordered structures, respectively (Carbonaro et al., 2012; Shevkani et al., 2019). Unordered structures quantified from CS-PI are twice as high

(9.48%) as those of ALKE-FBPI (3.47%), reflecting a difference in the industrial protein isolation method. The α-helix content among all protein isolates showed no significant differences, though DESE-FBPI had the highest amount, consistent with DES-extracted oat proteins in a previous study (Yue et al., 2021). The highest and lowest amounts of β-turns in CS-PI and DESE-FBPI, respectively, clearly indicated that the extraction method could induce alterations in β-structures. Unlike the conventional protein extraction mechanism, DES extraction is primarily determined by the hydrogen bond formation between proteins (amino and carboxylic groups) and solvent components (Lin et al., 2022). This unique interaction might also be modified by the amount of water present in the DES (Ferreira et al., 2021). Therefore, changes in the intermolecular interactions between the protein and DES could induce modifications to the secondary structure components.

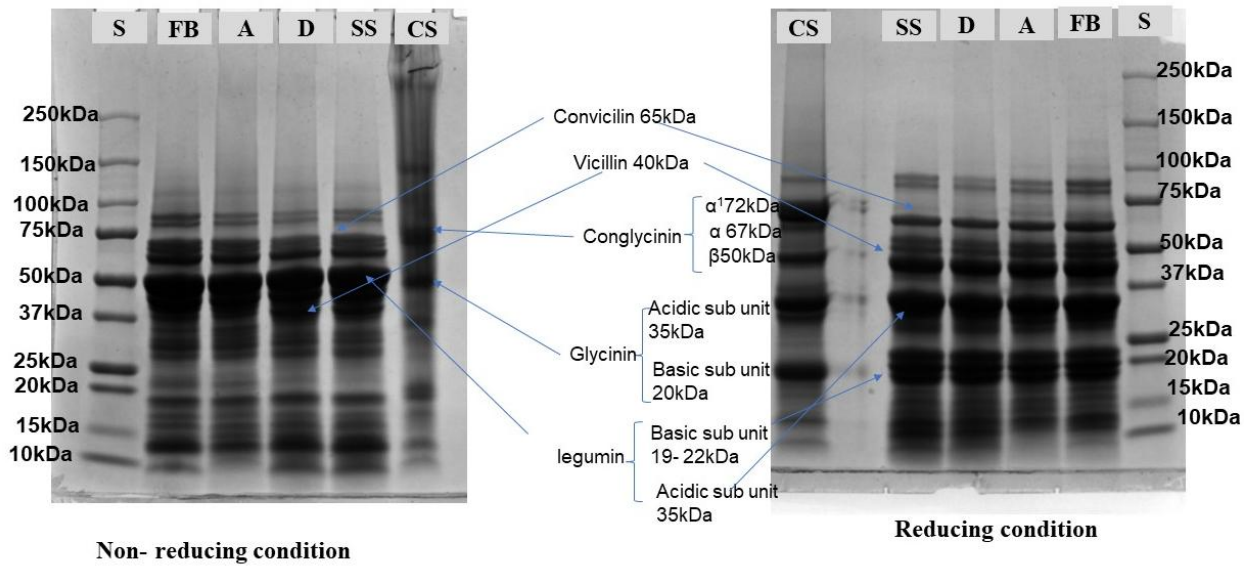

Figure 4.3. SDS-PAGE profile of protein isolates. Lane CS- Commercial soybean protein isolate (CS-PI), Lane SS- Salt-extracted fava bean protein isolate (SSE-FBPI), Lane D- Deep eutectic solvent-extracted fava bean protein isolate (DES-FBPI), Lane A-Alkaline-extracted fava bean protein isolate (ALKE-FBPI), Lane FB- Fava bean flour, and Lane S- molecular weight marker.

Table 4.5. The relative proportions of secondary structure components of extracted protein isolates

Secondary Structure Component	DES-FBPI		ALK-FBPI		SSE-FBPI		SC-PI	
	Peak center value (cm^{-1})	Relative content (%)	Peak center value (cm^{-1})	Relative content (%)	Peak center value (cm^{-1})	Relative content (%)	Peak center value (cm^{-1})	Relative content (%)
β-structures								
Anti-parallel β-sheets	1619	4.78	1681	5.1	1619	5.04	1625, 1681	22.03
β-strand	1627	6.89	1628	1 9.73	1629	10.13	-	-
β-sheets	1634, 1681	16.53	-	-	1640	7.45	-	-
β-turns	1667	5.22	1667	10.24	1165, 1679	14.91	1665	17.87
Total β structures		33.42		35.07		37.53		39.9
α-helix	1651, 1659	11.52	1650, 1660	9.82	1650, 1659	11.36	1651, 1659	9.5
Unordered structures	-	-	1643	3.47	-	-	1642	9.48
Intermolecular β sheets as aggregates (A*)	-	-	1692	4.93	1692	5.18	1693	6.53

Secondary structure components were analyzed using the amide I peak for deep eutectic solvent-extracted fava bean protein isolate (DESE-FBPI), Alkaline-extracted fava bean protein isolate (ALKE-FBPI), Salt-extracted fava bean protein isolate (SSE-FBPI), and Commercial soybean protein isolate (CS-PI): A*-Intermolecular β-sheet as aggregates.

4.5.3.4. Effect of extraction methods on protein subunit composition

As shown in Figure 4.3, SDS-PAGE was performed for all protein isolates under non-reducing and reducing conditions**.** Most of the polypeptide bands of fava bean protein isolates obtained from different extraction methods corresponded to legumin, vicilin, and convicilin. The electrophoretic profile obtained in this study is in agreement with the previously published results for fava bean proteins (Martinez et al., 2016; Vogelsang-O'Dwyer et al., 2020; Żmudziński et al., 2021). Under non-reducing conditions, the molecular weights of fava bean proteins, approximately ~37-43 kDa and ~42-51 kDa, likely correspond to vicilin and legumin, respectively. Above 60 kDa, a few polypeptide bands were observed in the 61-73 kDa range, with the 61 kDa band likely representing the convicilin fraction. Generally, the legumin fraction of fava bean comprises acidic (α-legumin) and basic (β-legumin) subunits linked by a disulfide bond (Sharan et al., 2021). Under reducing conditions, the bands observed in the 29-34 kDa and 17-21 kDa ranges corresponded to α-legumin and β-legumin, respectively. However, the band intensity of ALKE-FBPI (~42-51 kDa) for the legumin fraction was slightly reduced compared to that of DESE-FBPI and SSE-FBPI, whereas the albumin fraction, visible at ~10 kDa in the same lane, was completely absent. This was observed under both reducing and non-reducing conditions, indicating variations in protein composition induced by different extraction methods. Further, it suggests that protein isolates produced by acid precipitation are typically rich in globulin and, to a lesser extent, in albumin fraction. Besides, no substantial differences in protein composition were found between the fava bean protein isolates. The CS-PI mainly consists of β-conglycinin (or 7S globulin) and glycinin (or 11S globulin) along with a smaller amount of 15S and 2S albumin (Luthria et al., 2018; Verfaillie et al., 2023). β-conglycinin is composed of three subunits (α, α', and β) that are primarily associated through hydrogen bonds and hydrophobic interactions, whereas glycinin comprises an

acidic and a basic subunit linked by a disulfide bond (Verfaillie et al., 2023). Based on the protein profile of CS-PI under reducing conditions, the detectable bands at 72 kDa, 67 kDa, and 50 kDa were identified as α', α, and β-subunits of the 7S globulin, respectively (Nishinari et al., 2014). Three acidic subunits of the 11S may represent polypeptide bands of 31 kDa, 34 kDa, and 37 kDa, and one basic subunit of the 11S at 20 kDa (Chove et al., 2007). However, the bands at ~320 kDa and 164 kDa of CS-PI detected under non-reducing conditions were absent under reducing conditions. This likely corresponds to the disulfide cross-links formed by protein aggregation during high-temperature extraction (Zhang et al., 2022).

The 11S/7S ratios of all protein isolates were calculated based on the sum of the relative percentages of 11S (α-legumin + β-legumin) and 7S (convicilin + vicilin) proteins, as listed in Table 4.1S (Appendix 5). Our results are comparable to the findings of Jeganathan et al. (2023) for fava beans and soy proteins by Verfaillie et al. (2023). SSE-FBPI had the highest 11S/7S ratio (2.87), followed by DESE-FBPI (2.72) and ALKE-FBPI (2.48). As indicated by the protein profile of ALKE-FBPI, a slightly disintegrated legumin fraction may account for the lowest 11S/7S ratio. Between DES and SE, there was little difference in the 11S/7S ratio observed, likely due to the higher solubility of globulin in the salt environment. Overall, the results can be attributed to the fact that different isolation methods may extract varying proportions of protein subunits, such as the 11S and 7S fractions, which subsequently affect the amino acid composition and techno-functional properties.

4.5.3.5. Impact of extraction methods on the thermal stability of the protein isolates

The thermal properties of the extracted protein fractions, including onset temperature (T_o), peak transition temperature (T_d), and enthalpy change of the endotherm (ΔH), are presented in Table 4.3. The T_d value provides information about thermal stability of proteins, while ΔH reflects

the extent of ordered structure (Kudre et al., 2013; Wang et al., 2014). Thermal stability (T_d) of extracted protein fractions ranged from 126 to 138 °C, and no significant difference (P>0.05) was found among the proteins. The T_d values obtained in our study are higher than the reported values for fava bean proteins (Sharan et al., 2022). This may be due to the use of dried protein powder, the low moisture content, and the high purity of protein isolates (Ricci et al., 2018). Especially in this study, the protein samples were dried under P_2O_5, resulting in extremely dry samples. In the case of enthalpy changes (ΔH), it was significantly varied among the protein isolates; both DESE-FBPI and SSE-FBPI showed significantly higher ($P < 0.05$) endothermic peaks compared to either ALKE-FBPI or CS-PI. The T_d and ΔH of proteins can be associated with factors such as amino acid composition, protein structural conformation, and residual salt added by processing methods (Mir et al., 2019; Tang & Sun, 2011). The thermal stability of proteins is believed to be determined by the proportion of hydrophobic amino acid residues and a higher degree of β-sheets (Gundogan & Can Karaca, 2020; Ju et al., 2023). As minor differences were observed in the amino acid content (Table 4.7) and secondary structural components (Table 4.5) of all the protein isolates, thermal stability might not have changed significantly. Nonetheless, the extent of the ordered structure of proteins obtained from different extraction methods seemed profound. This was particularly evident with the content of unordered structures and protein aggregates present in ALKE-FBPI and CS-PI (Table 4.5). Therefore, the endotherm peak (ΔH) was significantly higher in DESE-FBPI and SSE-FBPI. This suggests that DES extraction may produce proteins with less denaturation, without significant alteration in their secondary structure components. In a recent study, Chen et al. (2021) reported that DES-extracted soy protein exhibited better heat resistance and stronger hydrophobicity compared to commercial soy proteins. The unique interaction between proteins and the DES matrix, mediated by the hydrogen bond network, may play a crucial

role in determining proteins' thermal stability and conformational changes. Further, the substitution of water with the DES system may have a significant impact on the structural changes of the protein. However, further studies are warranted to unravel the mechanism of the thermal stability of DES-extracted proteins.

4.5.4. Effect of protein extraction methods on functional properties of fava bean protein isolates

4.5.4.1. Solubility

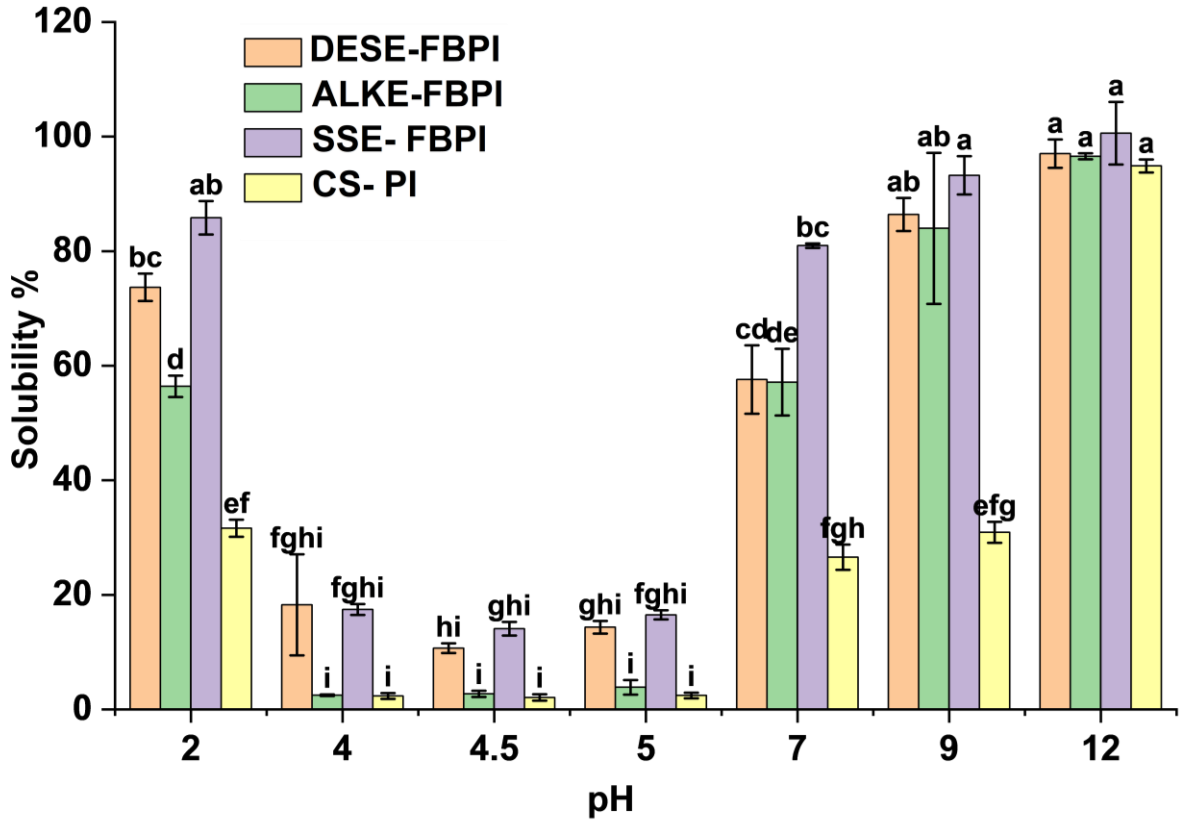

Figure 4.4. Solubility of the extracted protein isolates as a function of pH; DESE-FBPI- Deep eutectic solvent-extracted fava bean protein isolates, ALKE-FBPI- Alkaline-extracted fava bean protein isolate, SSE-FBPI- Salt-extracted fava bean protein isolate, and CS-PI- Commercial soybean protein isolate. All data are presented as means ± standard deviations and were analyzed

by analysis of variance (ANOVA) followed by Tukey's test (n=3). Different letters on the error bars indicate a significant difference at $P < 0.05$.

The solubility of proteins plays an important role in a wide range of food applications. It is a prerequisite for other functional properties such as foaming, emulsification, and gelation. The solubility of proteins primarily depends on the balance between protein-protein and protein-solvent interactions (Shevkani et al., 2019). Proteins often exhibit minimum solubility at the isoelectric pH (pI) due to protein-protein interactions, whereas above and below pI, solubility increases due to higher electrostatic repulsion between protein molecules. The solubility profile of protein isolates produced from different extraction methods is shown in Figure 4.4. The solubility profile of all protein isolates appeared to be U-shaped, consistent with previously reported data (Krause et al., 2023; Vogelsang-O'Dwyer et al., 2020). The highest solubility was observed for all protein isolates at high acidity or alkaline conditions, while the lowest was at the pI (pH 4.0-5.0). Overall, except at pH 12.0, the SSE-FBPI exhibited significantly higher solubility ($P < 0.05$) across the studied pH range, and this solubility was reduced in the order of DESE-FBPI > ALKE-FBPI > CS-PI. Except for the DESE-FBPI and SSE-FBPI, the hydrophobicity (S_0) results obtained for the protein isolates in this study (Table 4.3) directly correlated with the solubility, plausibly due to the extent of denaturation. This means that the higher the S_0, the lower the solubility. As can be seen, although S_0 of DESE-FBPI was significantly lower than that of SSE-FBPI (Table 4.3), the corresponding solubility values were opposite. Protein solubility largely depends on the balance of hydrophilic and hydrophobic amino acids (AAs) and the charge distribution on the protein surface. The higher amount of hydrophobic AA content in DESE-FBPI, combined with a lower ratio of hydrophilic to hydrophobic AAs, may likely contribute to the lower solubility compared to SSE-FBPI (Table 4.7). Based on a recent study, oat protein extracted from a butanediol-based

DES/water system was found to have relatively poor solubility (<25%), despite its high content of hydrophilic AA (Yue et al., 2021). The authors suggest that the secondary structure components, β-sheets, and β-turns in DES-extracted oat proteins were also responsible for protein solubility. Moreover, DESE-FBPI and SSE-FBPI exhibited significantly higher solubility in the pI region (pH 4.0-5.0), ranging from 10% to 16%, compared to the other two isolates (2-2.8%). This implies that protein-protein interactions (hydrophobic) were higher in both ALKE-FBPI and CS-PI than in the DESE-FBPI and SSE-FBPI at the pI. The effect of AE on protein solubility was more pronounced as both ALKE-FBPI and SC-PI exhibited significantly lower solubility than that of protein isolates obtained from DES and SE. Commercial protein isolates especially often exhibit lower solubility due to the high degree of denaturation during the thermal treatment employed in the industrial process (Monteiro & Lopes-da-Silva, 2019). This was evident in our study with the CS-PI, as it had the lowest solubility among all other protein isolates; this trend is consistent with other studies on CS-PI (Paredes-Lopez et al., 1991; Tang et al., 2021). In contrast, Karaca et al. (2011) reported a higher solubility (89.65%) of ALKE-FBPI compared to SSE-FBPI (52.54%) at pH 7.0 under laboratory extraction conditions. However, in another study (Gundogan et al., 2020), pea protein isolates obtained from SE showed higher solubility than those from AE.

4.5.4.2. Water (WHC) and Oil (OHC) holding capacity

The WHC and FHC provide information about the amount of water and oil that can be absorbed per gram of protein (Boye et al., 2010). These are important properties related to solid foods, particularly in plant-based meat analogs, that influence perceived juiciness. Figure 4.5 illustrates the WHC and OHC of protein isolates obtained from various protein extraction methods. WHC varied significantly ($P < 0.05$) depending on the isolation technique. The highest WHC was exhibited by CS-PI and ALKE-FBPI, with no significant difference ($P>0.05$), followed by DESE-

FBPI and SSE-FBPI, among which SSE-FBPI had a significantly lower WHC than DESE-FBPI (P<0.05) . It was expected that the higher the S_0 of protein isolates (Table 5.3), the lower the WHC. Instead, we observed the opposite trend in this study. This was further confirmed by the hydrophilic AA content of the protein isolates (Table 4.7). Water binding to proteins occurs through various intermolecular interactions, including ion-dipole, dipole-dipole, and hydrophobic interactions. Notably, the microstructure of the protein matrix can determine the association of water with the protein (Lam et al., 2018).

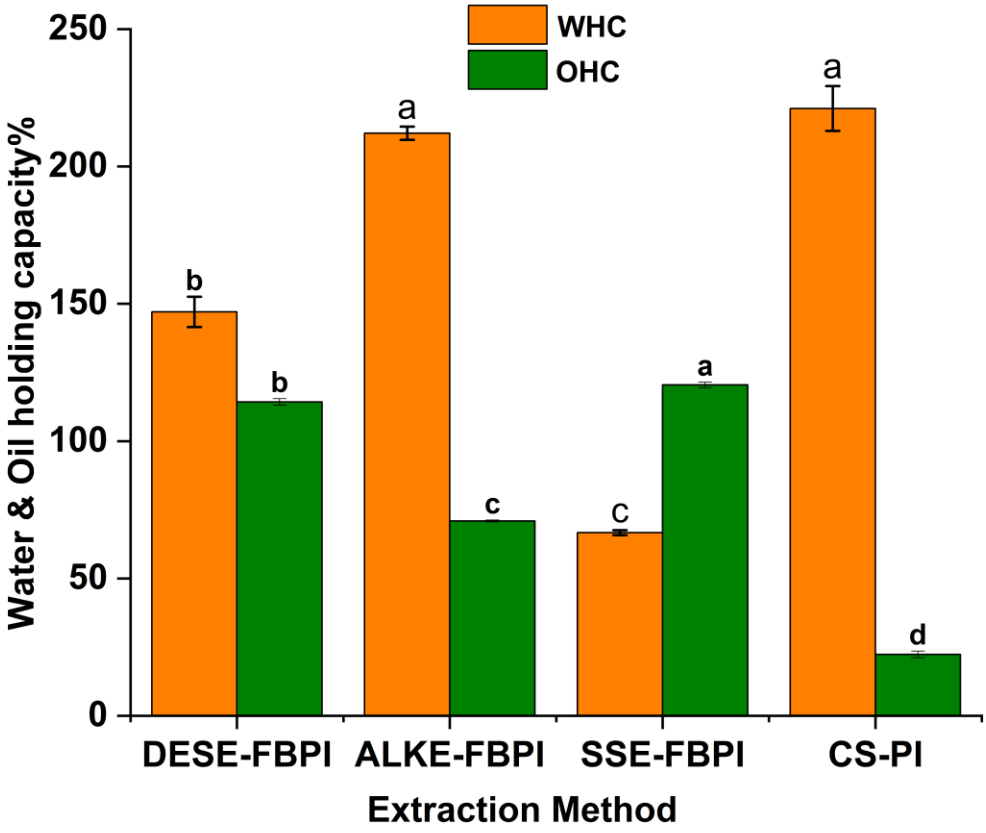

Figure 4.5. Water-holding (WHC) and Oil-holding capacity (OHC) of protein isolates obtained from different extraction methods. DESE-FBPI, Deep eutectic solvent-extracted fava bean protein isolate; ALKE-FBPI, Alkaline-extracted fava bean protein isolate; SSE-FBPI, Salt-extracted fava

bean protein isolates; and CS-PI, Commercial soybean protein isolate. All data were analyzed by analysis of variance, followed by Tukey's test (n=3). Different letters on error bars indicate a significant difference at $P < 0.05$.

Arrese et al. (1991) reported that a high degree of denatured soybean proteins affected the water imbibing capacity more than the native state. Thus, unfolded polypeptide chains may probably be required to obtain a protein matrix capable of entrapping a significant amount of water (Arrese et al., 1991). Additionally, differences in the initial moisture content of protein isolates (Table 4.2) may also contribute to the observed pattern in WHC. Furthermore, physiochemical environments such as pH and ionic strength also significantly affect the water-binding properties of proteins (Zhang et al., 2021). Similar observations were reported for commercially produced pea and soybean protein isolates (Fuhrmeister & Meuser, 2003; Stone et al., 2014). Pea protein extraction by micellization led to higher WHC (3.2-3.6 g/g), followed by alkaline (2.4-2.6 g/g) and salt (0.34-2.6 g/g) extractions (Stone et al., 2014). Compared to the literature, the WHC values obtained in this present study for ALKE-FBPI are relatively higher and lower than those reported by Vioque et al. (2012), and Krause et al. (2023), respectively.

OHC is a crucial factor in the distribution and emulsification of oil in specific food applications, such as mayonnaise, meat analogs, and spreads. (de Paiva Gouvêa et al., 2023). Interestingly, in the case of OHC, all protein isolates exhibited the complete opposite trend to WHC. Significantly higher FHC in SSE-FBPI was observed compared to others, in the following order: DES-FBPI > ALKE-FBPI > CS-PI. As described earlier, since the highest S_o was found with CS-PI (Table 4.3), it was generally expected to have a higher OHC as fat interacts with the non-polar side chain of proteins (Sathe et al., 1982). Similar to the WHC of protein isolates, the microstructure of proteins can impact oil entrapment, allowing them to bind and retain a significant

amount of oil in their hydrophobic core (Kinsella, 1979). Furthermore, protein isolates with small particle sizes and larger surface areas can entrap more oil than larger sizes, which is comparable to the data obtained for particle size (Table 4.4) in this study. Therefore, the smaller particle size of the DESE-FBPI and SSE-FBPI may also have contributed to the higher FHC. Stone et al. (2014) found that the extraction method was a significant factor in determining OHC in pea proteins, with higher values in SE proteins than in AE or micellization. The OHC value obtained for ALKE-FBPI in the current study is lower than previously reported for fava beans (Eckert et al., 2019; Vioque et al., 2012). Overall, differences in surface properties of isolates, cultivars, and/or methodologies adopted for measuring WHC and OHC may also have contributed to the variations in WHC and OHC among the protein isolates.

4.5.4.3. Emulsification activity index (EAI) and stability index (ESI)

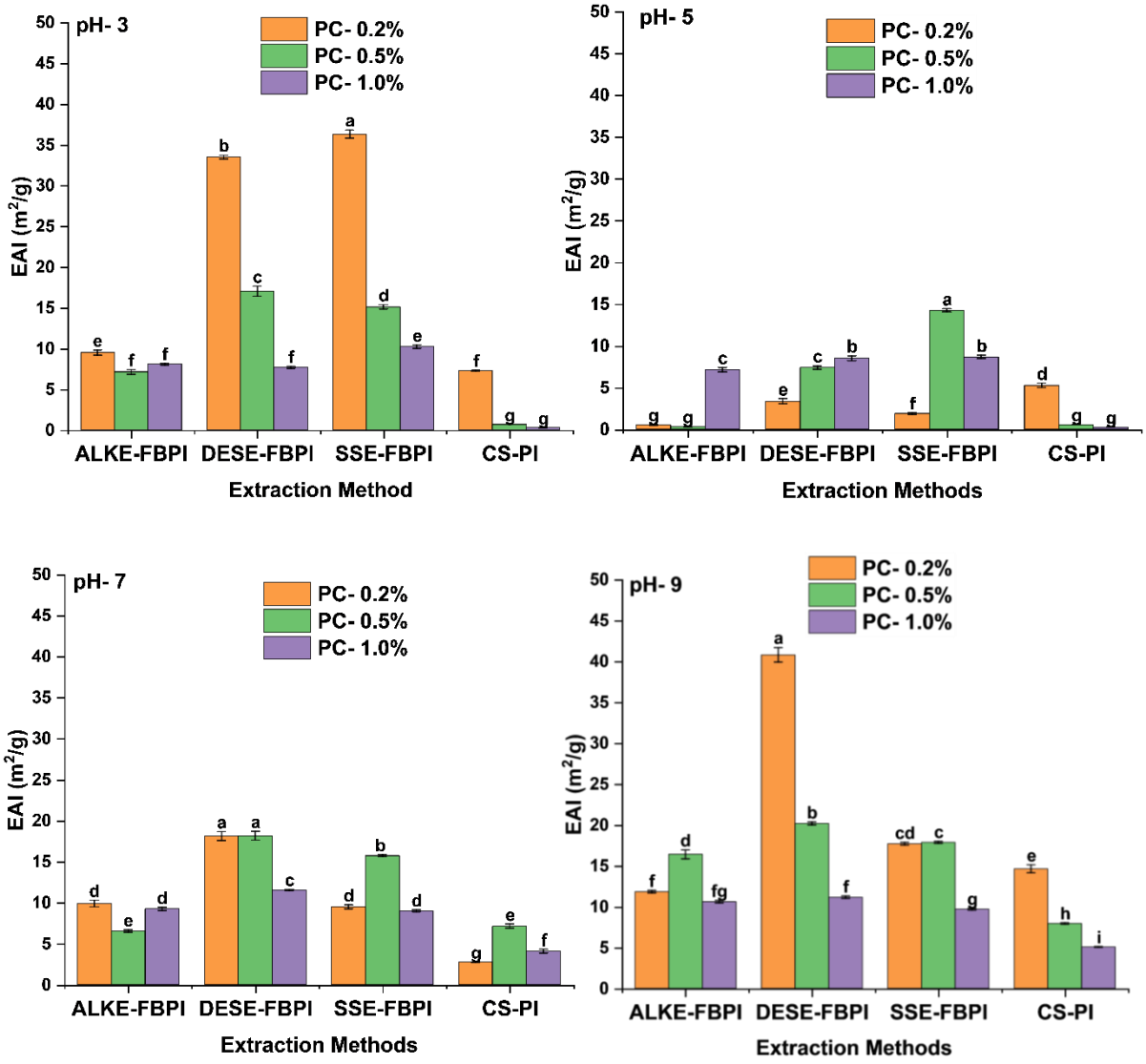

Figure 4.6A. Emulsification activity index (EAI) of extracted protein isolates at different pH (3.0, 5.0, 7.0, 9.0) and protein concentrations (0.2, 0.5, 1.0% w/v). DESE-FBPI, Deep eutectic solvent-extracted fava bean protein isolate; ALKE-FBPI, Alkaline-extracted fava bean protein isolate; SSE-FBPI, Salt-extracted fava bean protein isolate; and CS-PI, Commercial soybean

protein isolate. All data were analyzed by analysis of variance followed by Tukey's test (n=3), and different letters on error bars indicate a significant difference at $P < 0.05$.

An emulsion is generally formed by mixing two immiscible liquids, in which one is dispersed as small droplets in the continuous phase (Lam et al., 2018). Proteins can adsorb to the oil-water interface to minimize the interfacial energy between the two phases due to their amphiphilic nature (Keivaninahr et al., 2021). The emulsification activity index (EAI) estimates the oil-water interface that a unit weight of protein can stabilize, based on the turbidity in a diluted emulsion. In contrast, the emulsification stability index (ESI) measures the ability to withstand changes in emulsion structure over a defined period (Pearce & Kinsella, 1978). The effect of different extraction methods on EAI and ESI measured at various pH values (3.0, 5.0, 7.0, and 9.0) and protein concentrations (PC) (0.2, 0.5, and 1% w/v) is presented in Figures 4.6A and 4.6B, respectively. The variance analysis revealed that the extraction method had a significant impact on both EAI and ESI ($P < 0.05$). For DESE-FBPI, an increase in EAI was observed with increasing PC at pH 5.0, and no such trend was observed at pH 7.0. However, at pH 3.0 and 9.0, there was a decreasing trend in the EAI as the PC increased. In general, acidic and alkaline media were favored for the higher EAI of most protein isolates produced, with the latter being the most preferred environment. This is due to the higher solubility at alkaline pH, resulting from greater electrostatic repulsion between proteins. Previous studies reported similar behavior for fava beans (Żmudziński et al., 2021), kidney beans, and pea proteins (Shevkani et al., 2015). In our study, SSE-FBPI had significantly higher EAI, followed by DESE-FBPI at pH 3.0 with a PC of 0.2% (w/v), corresponding to the highest solubility (Figure 4.4) and the OHC (Figure 4.5). With higher solubility, proteins can easily migrate and adsorb at the interface to form a viscoelastic film around the oil droplets. Moreover, the significantly reduced particle size of SSE-FBPI, compared to other

cases (Table 4.4), may also promote better interfacial activity due to its fast mobility toward the water-oil interface. Despite the lower S_0 of SSE-FBPI (Table 4.3), the molecular flexibility of SSE-FBPI may enhance its greater EAI, making it easier to reorient at the interface, as verified by the higher content of the low molecular weight vicilin fraction analyzed by the SDS-PAGE (Table 4.1S- Appendix 5). Kimura et al. (2008) reported that Vicilins from peas exhibited superior emulsifying properties compared to the legumin fraction, attributed to their greater solubility and higher surface hydrophobicity. However, in another study, kidney bean proteins exhibited a higher EAI than those from pea proteins, despite having lower hydrophobicity and protein solubility (Shevkani et al., 2014). At the pI, protein-protein interaction is dominant due to zero net charges; thus, protein solubility is minimal. This was reflected at pH 5.0, where all protein isolates had lower EAI than at the other pH values.

In contrast, EAI was significantly higher in DESE-FBPI at both alkaline and neutral pH levels than in SSE-FBPI, with the greatest EAI observed at alkaline pH. The lower hydrophilic: hydrophobic ratio found in DES-FBPI, compared to SSE-FBPI (Table 4.3), reflected the presence of more hydrophobic groups on the surface. This leads to a considerable decrease in the interfacial energy barrier, increasing the better adsorption of protein molecules at the interface. Also, the greater electrostatic repulsion between protein molecules in alkaline media enhances adsorption at the oil-water interface. Additionally, the lower number of β-sheets and the absence of intermolecular β-sheets, as observed in DESE-FBPI compared to other protein isolates (Table 4.5), may be conducive to the improved flexibility of polypeptides, leading to greater adsorption at the oil/water interface. Moreover, for ALKE-FBPI and CS-PI, EAI values were significantly lower (<20 m^2/g) than those of the isolates prepared by salt and DES extractions across all pH values. This may be attributed to the lower solubility, as seen in Figure 4.4, despite the higher hydrophobicity ($P < 0.05$)

(Table 4.3) compared to those with DES-FBPI and SSE-FBPI. Typically, the higher the β-sheet content of proteins, the lower their molecular flexibility (Ding et al., 2020). The higher number of β-sheets found in CS-PI (Table 4.5) and the presence of disulfide cross-links (Figure 4.3) may prevent the proteins' reorientation at the interface. Karaca et al. (2011) reported that proteins produced from chickpeas, fava beans, lentils, and peas using SE were less effective in forming emulsions at pH 7.0 compared to the acid precipitation method, and EAI was found to be positively correlated with protein surface charge and solubility.

Except in CS-PI, the ESI of the prepared protein isolates did not show considerable variations within the pH and PC ranges investigated (Figure 4.3B). CS-PI showed significantly higher ESI than other protein isolates across all pH ranges, regardless of the PC. Among the ESI values of CS-PI, the stability of the emulsion at acidic pH (3.0) was the greatest, lasting ~80 min, followed by the values at pH 5.0 (~66 min) with PC of 0.5 and 0.2% (w/v), respectively. The disulfide cross-links detected in SDS-PAGE (Figure 4.3) for the CS-PI likely contributed to stabilizing the emulsion, along with the soybean protein processing, which induced the formation of aggregates that created a thicker mechanical protection layer, preventing oil droplets from coalescing (San et al., 2025). On the other hand, the high amount of β-sheets (Table 4.5) in the CS-PI compared to other protein isolates promotes interaction between adjacent protein molecules, resulting in greater stability and thereby reducing the interfacial tension of the oil-water interface (San et al., 2025). However, at pH 7.0 and 9.0, ESI was substantially reduced to ~ 20 min. Although protein solubility increases at alkaline pH values more than in acidic environments, protein molecules at the oil-water interface may not be able to form a cohesive, strong viscoelastic film between neighboring molecules due to unfavorable surface charges. Hence, both hydrophilic and

hydrophobic interactions are vital; that is, the net charge on proteins should be large enough to stabilize the electrostatic repulsive forces between oil droplets (Karaca et al., 2011).

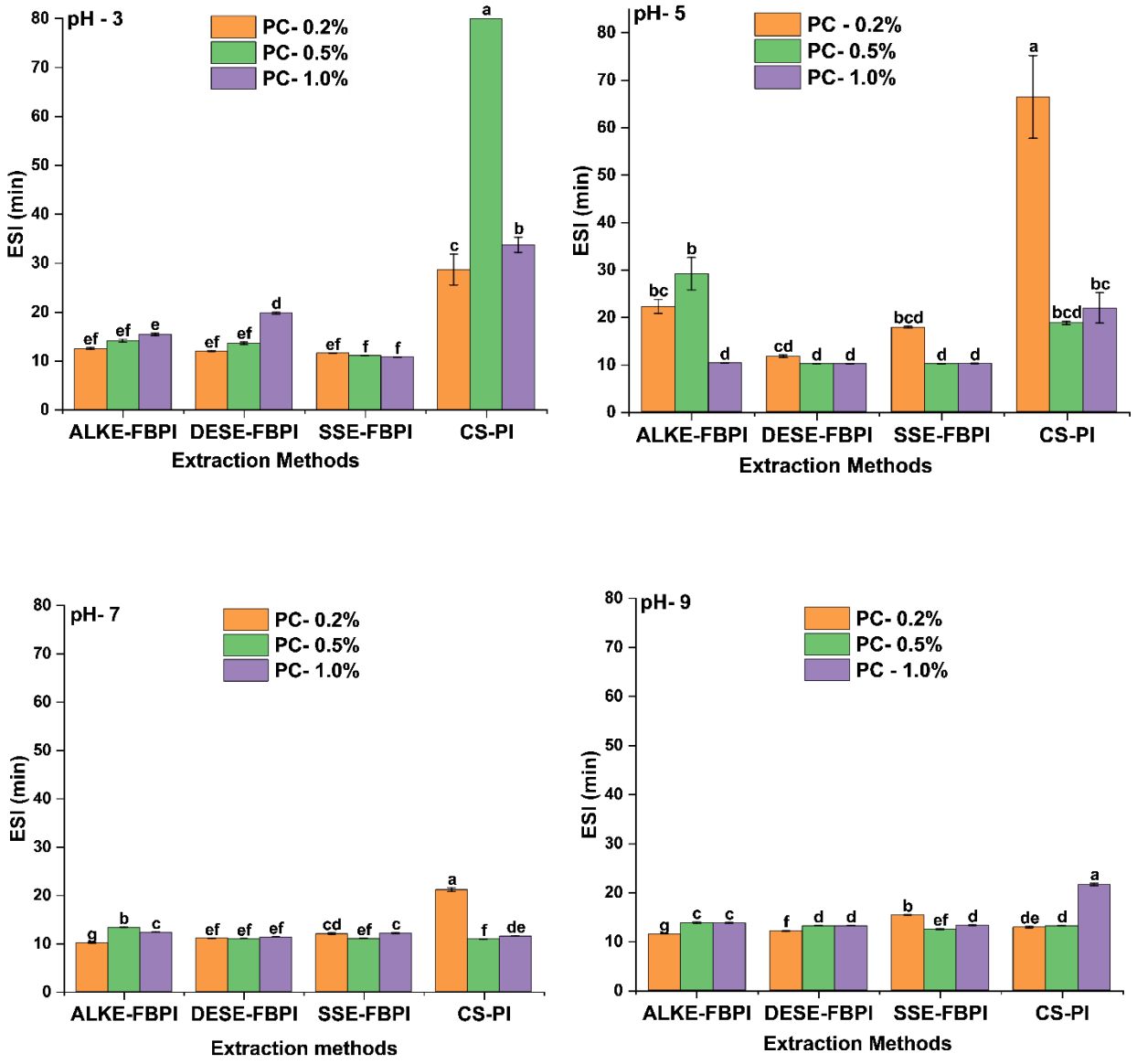

Figure 4. 6B. Emulsification stability index (ESI) of extracted protein isolates at different pH (3.0, 5.0, 7.0, 9.0) and protein concentrations (0.2, 0.5, 1.0% w/v). DESE-FBPI, Deep eutectic solvent-extracted fava bean protein isolate; ALKE-FBPI, Alkaline-extracted fava bean protein isolate; SSE-FBPI, Salt-extracted fava bean protein isolate; and CS-PI, Commercial soybean protein

isolate. All data were analyzed by analysis of variance followed by Tukey's test (n=3), and different letters on error bars indicate a significant difference at $P < 0.05$.

The emulsion stability, affected by other protein isolates obtained from DES, alkaline, and salt extraction methods, showed slight variations, with ESI values ranging from 10 to 20 min across all pH ranges studied. The differences in EAI and ESI values in the present study may reflect variations in the physicochemical properties of proteins induced by the different extraction methods.

4.5.4.4. Foaming capacity (FC) and stability (FS)

The foaming properties (FC, FS) are important in various food applications, including whipped cream, mousse, ice cream, beverages, bread, and cakes. FC measures the amount of foam produced per volume of protein solution through air incorporation, while FS is the ability of the protein system to stabilize the foam over some time before it collapses (Ma et al., 2022). The results of the FC and FS of all protein isolates measured at various pH and PC are presented in Figures 4.7A and 4.7B. Analysis of variance revealed that extraction methods were significant factors ($P<0.05$) in determining the FC. FC was higher when the PC was increased in all protein isolates, and the increase was more pronounced at acidic pH values. At higher PC, protein adsorption is rapid at the air-water interface due to the higher intensity of protein diffusion (Zayas, 1997). A similar trend was reported with ALKE-FBPI at a PC of 1-5% (w/v) and a pH range of 5.0-8.0 (Żmudziński et al., 2021). The authors demonstrated that increased PC and surface tension were significantly reduced, positively affecting the FC. Generally, better FC is expected when proteins are away from their pI due to increased protein net charges (Gochev et al., 2014). However, our study observed that all protein isolates at pH 3.0 and 5.0 had better FC than those at pH 7.0 and 9.0. This may be attributed to the protein's inter- and intra-protein molecular

interactions, with greater hydrophobic/hydrophilic charge balance dominated by lower pH. DESE-FBPI had a significantly higher FC than other protein isolates at all pH values with 1% (w/v) PC. Protein solubility is one of the prerequisite factors for better foaming ability (Amagliani et al., 2021). Although we observed that DESE-FBPI's solubility was inferior to SSE-FBPI, DESE-FBPI may still possess an enhanced ability to reorient at the air-water interface at a faster rate, possibly due to its higher hydrophobic: hydrophilic ratio, as confirmed by amino acid composition analysis (Table 4.7). Moreover, for CS-PI, FC was similar to DESE-FBPI ($P>0.05$) at pH 7.0 with 0.5% (w/v) PC. However, in general, the FC of CS-PI was significantly lower than those of other protein isolates at most pH values and PC. In the commercial soybean protein isolation process, the aggregated globulin can lead to a larger self-assembled state, slowing the diffusion rate and reducing the adsorption rate at the interface, thereby affecting the FC (Yang & Sagis, 2021). After the DESE-FBPI, the second-highest FC ($P<0.05$) was observed with ALKE-FBPI at all pH values (except at pH 9.0), with a PC of 1% (w/v). SSE-FBPI had a similar FC to ALKE-FBPI at neutral pH, and this trend was further observed at pH 3.0 and 5.0, with PC values of 0.5% and 0.1%, respectively. Notably, the interfacial behavior of proteins isolated from SE differed at pH 5.0 and 9.0, compared to all other protein isolates, wherein increasing PC led to significantly reduced FC. Higher PC is expected to have increased adsorption at the air/water interface, but it did not reflect in SSE-FBPI (at pH 5.0 and 9.0). The higher legumin to vicilin ratio (Table 4.1S-Appendix 5) and the lower hydrophobicity (Table 4.3) of SSE-FBPI compared to other counterparts may have restricted the proteins from being adsorbed efficiently at the air-water interfacial membrane.

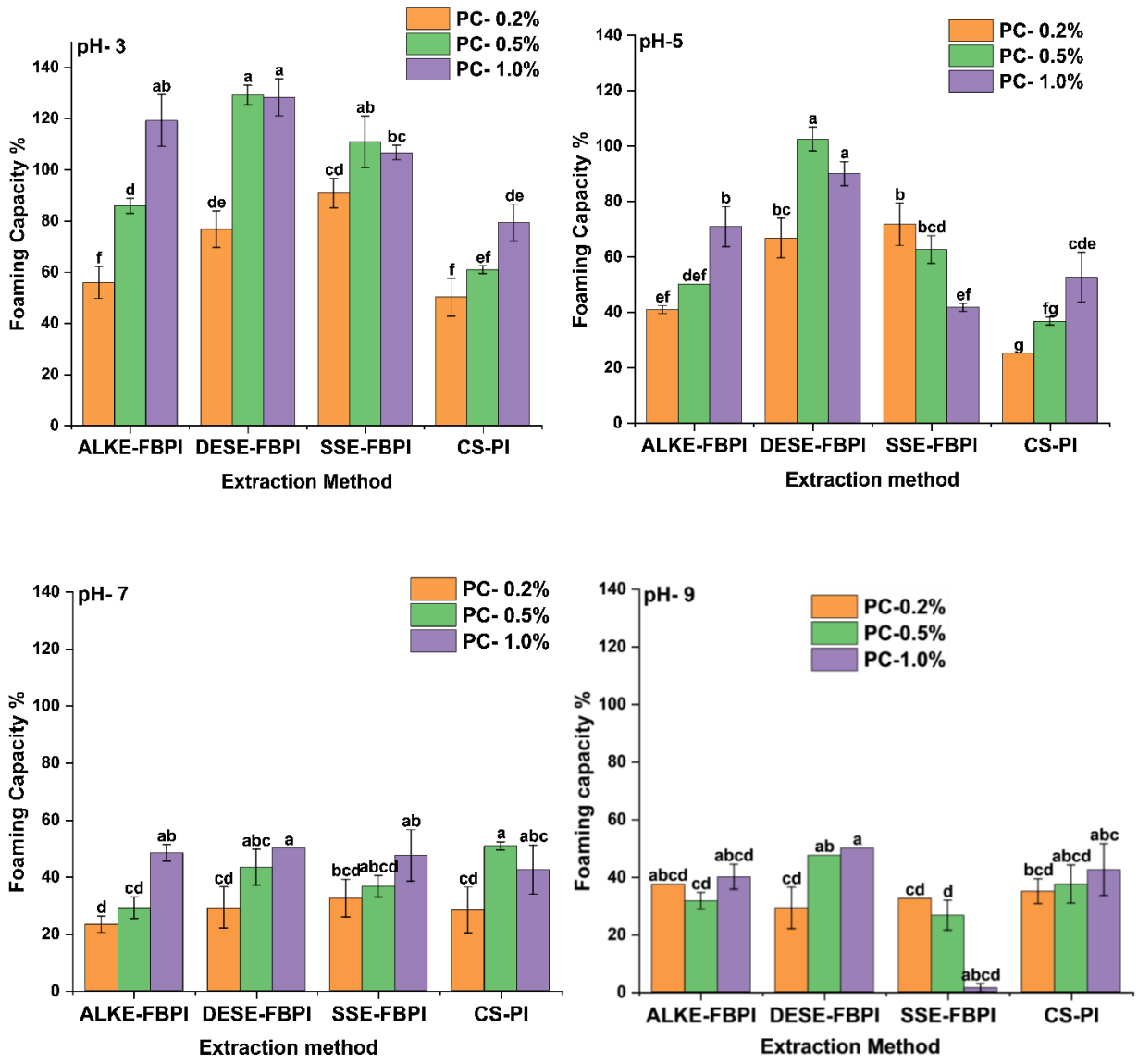

Figure 4. 7A. Foaming capacity (FC) of extracted protein isolates at different pH (3.0, 5.0, 7.0, 9.0) and protein concentration (0.2, 0.5, 1.0% w/v). DESE-FBPI, Deep eutectic solvent-extracted fava bean protein isolate; ALKE-FBPI, Alkaline-extracted fava bean protein isolate; SSE-FBPI, Salt-extracted fava bean protein isolate; and CS-PI, Commercial soybean protein isolate. All data were analyzed by analysis of variance followed by Tukey's test (n=3), and different letters on error bars indicate a significant difference at $P< 0.05$.

FS was also affected by extraction methods ($P<0.05$) evaluated at different PC and pH, and the results are presented in Figure 4.7B. Generally, at pH levels of 3.0 and 5.0, the FS decreased as PC increased from 0.2% to 0.5%. However, with further increases in PC, there was a minimal effect on the FS. At higher protein concentrations, protein interactions may be greater, forming a thick layer around the air-water interface and enhancing foaming stability (Amagliani et al., 2021). However, a certain degree of electrostatic repulsion induced by the pH may enhance contact between air bubbles, leading to coalescence (Dombrowski et al., 2018). Conversely, for a PC of 0.5%, the foam was stabilized at a higher level for most protein isolates in both neutral and alkaline environments. This indicates that FS is primarily influenced by both PC and pH. FS for ALKE-FBPI was significantly higher (~80%) in an acidic environment (pH 3.0 and 5.0) with a PC of 0.2%. in comparison, both ALKE-FBPI and CS-PI exhibited similar FS (~85-100%) at neutral and alkaline pH values. This can be attributed to the significantly higher surface hydrophobicity (Table 4.3) of ALK-FBPI and CS-PI compared to other proteins extracted, which may facilitate better reorientation at the air-water interface, resulting in a stronger film around the air bubbles. In agreement with the present study, Żmudziński et al. (2021) reported better FS for ALKE-FBPI at 1% PC and pH 6.8. than the FS recorded at pH 5.0 and 8.0 with PC of 5% and 8%. The author attributed the higher FS to the larger hydrodynamic radius of the particles and the presence of both hydrophilic and hydrophobic zones. DESE-FBPI exhibited significantly lower FS than other protein isolates at pH 7.0 and 9.0. Secondary structure components are believed to be responsible for the arrangement of the polypeptide chain at the interface, mainly including α-helix, β-turn, β-sheets, and random coils (Li et al., 2021). The lower surface hydrophobicity (Table 4.3) and the lower secondary structure components, specifically β-sheets (Table 4.5), compared to other cases, may have a negative impact on the FS of DESE-FBPI. However, the foam remained generally

stable at ~70-80%. Except at pH 3.0, SSE-FBPI showed significantly higher FS (~70-85%) across all other pH levels compared to the protein obtained through DES extraction. For the CS-PI, and regardless of the PC used, FS was significantly lower (<10%) at pH 5.0 compared to all other protein isolates. The minimum solubility of CS-PI at the pI may be associated with the poor FS at pH 5.0. Furthermore, similar to previous literature reports on pea proteins (Stone et al., 2014) and lentils (Osemwota et al., 2021), the results from the present study have shown that different extraction methods are significant factors in determining FC and FS.

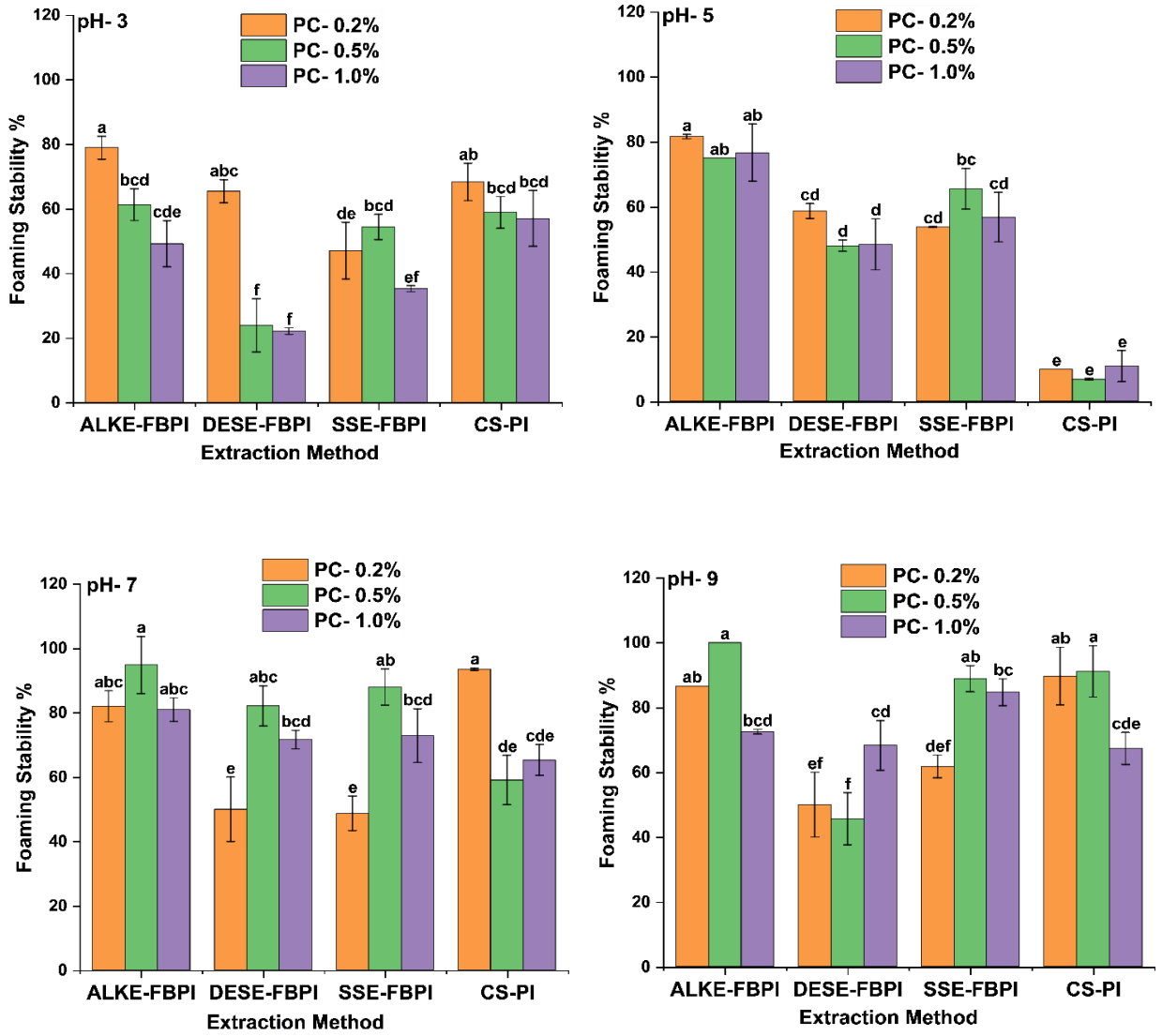

Figure 4. 7B. Foam stability (FS) of extracted protein isolates at different pH (3.0, 5.0, 7.0, 9.0) and protein concentration (0.2, 0.5, 1.0% w/v). DESE-FBPI, Deep eutectic solvent-extracted fava bean protein isolate; ALKE-FBPI, Alkaline-extracted fava bean protein isolate; SSE-FBPI, Salt extracted-fava bean protein isolate; and CS-PI, Commercial soybean protein isolate. All data were analyzed by analysis of variance followed by Tukey's test (n=3), and different letters on error bars indicate a significant difference at $P< 0.05$.

4.5.4.5. Least gel concentration (LGC)

Protein gelation is essential in various food applications, including tofu, cheese, yogurt, and meat analogs. In gelation, protein is partially or fully denatured upon heating, exposing hydrophobic residues that then aggregate to form a continuous three-dimensional protein network at a sufficient protein concentration (Zha et al., 2021). Protein aggregation occurs through interactions among the exposed active side chains, including non-covalent interactions (such as hydrogen bonds, hydrophobic interactions, and electrostatic interactions) and covalent interactions (such as disulfide bonds) (Nicolai & Chassenieux, 2019). The gelling process can be affected by various factors, including PC, pH, ionic strength, and extraction method (Langton et al., 2020; Nicolai et al., 2109). Protein extraction methods play a crucial role in determining the gelling properties, as the choice of solvent, pH, and temperature can significantly influence the proportions of different protein fractions (Langton et al., 2020). The LGC of all protein isolates obtained at different PC (5-20%, w/v) and pH values (3.0, 5.0, 7.0, and 9.0) are presented in Table 4.6. The gelling behavior of protein isolates from different methods varied. The LGC of DESE-FBPI was low (5%, w/v) at pH 3.0, while higher PC was required for DESE-FBPI to form self-standing gels at pH 5.0 (10% w/v), 7.0 (10% w/v), and 9.0 (12% w/v). A similar trend was also observed with other protein isolates; ALKE-FBPI had an LGC of 5% (w/v) and 12% (w/v) at both pH 3.0 and 5.0,

and pH 7.0 and 9.0, respectively. The value obtained at pH 7.0 for ALKE-FBPI aligns with the reported studies (Nilsson et al., 2022; Vogelsang-O'Dwyer et al., 2020). CS-PI and SSE-FBPI, on the other hand, showed similar LGC (8% w/v) at lower pH conditions (pH 3.0 and 5.0). In alkaline conditions, proteins become more soluble than in other pH values (Figure 4.4), which may require higher PC to form self-supporting gels. Therefore, a similar LGC (12% w/v) was observed for all protein isolates at pH 9.0. For CS-PI, the lowest LGC recorded at pH 7.0 was 5% (w/v), which is similar to the values reported by Ningtyas et al. (2021). Interestingly, based on the LGC of all protein isolates, only CS-PI and DESE-FBPI could form firm gels (able to be removed from the tube without breaking) at pH 7.0, with PC values of 12-20% and 20%, respectively. Thus, DESE-FBPI shows potential for food applications such as tofu and meat analogs, similar to soybean proteins.

Table 4. 6. Least gel concentration (LGC) of protein isolates extracted using different methods.

pH	3						5						7						9					
Ingredients	5 %	8 %	10 %	12 %	15 %	20 %	5 %	8 %	10 %	12 %	15 %	20 %	5 %	8 %	10 %	12 %	15 %	20 %	5 %	8 %	10 %	12 %	15 %	20 %
FBF	S	S	S	S	F	F	F	F	F	F	F	F	F	F	F	F	F	F	F	F	F	F	F	F
DES-FBPI	S	S	S	S	S	S	×	×	S	S	S	S	×	×	S	S	S	F	×	×	×	S	S	S
ALK-FBPI	S	S	S	S	S	S	S	S	S	S	S	S	×	×	×	S	S	S	×	×	×	S	S	S
Salt-FBPI	×	S	S	S	S	S	×	S	S	S	S	S	×	×	S	S	S	S	×	×	×	S	S	S
Soy- PI	×	S	S	S	S	S	×	S	S	S	S	S	S	S	S	F	F	F	×	×	×	S	F	F

Least gel concentration (LGC) of DESE-FBPI (Deep eutectic solvent-extracted fava bean protein isolate), ALKE-FBPI (Alkaline extracted-fava bean protein isolate), SSE-FBPI (Salt-extracted fava bean protein isolate) and CS-PI (Commercial soybean protein isolate at different protein concentration (5-20% w/v) and pH (3.0-9.0). The gels formed were categorized as firm (F), when the gel could be removed from the tube without breaking, and soft gels (S) when it fell apart. × denotes no gel formation.

4.5.5. Effect of extraction method on protein quality

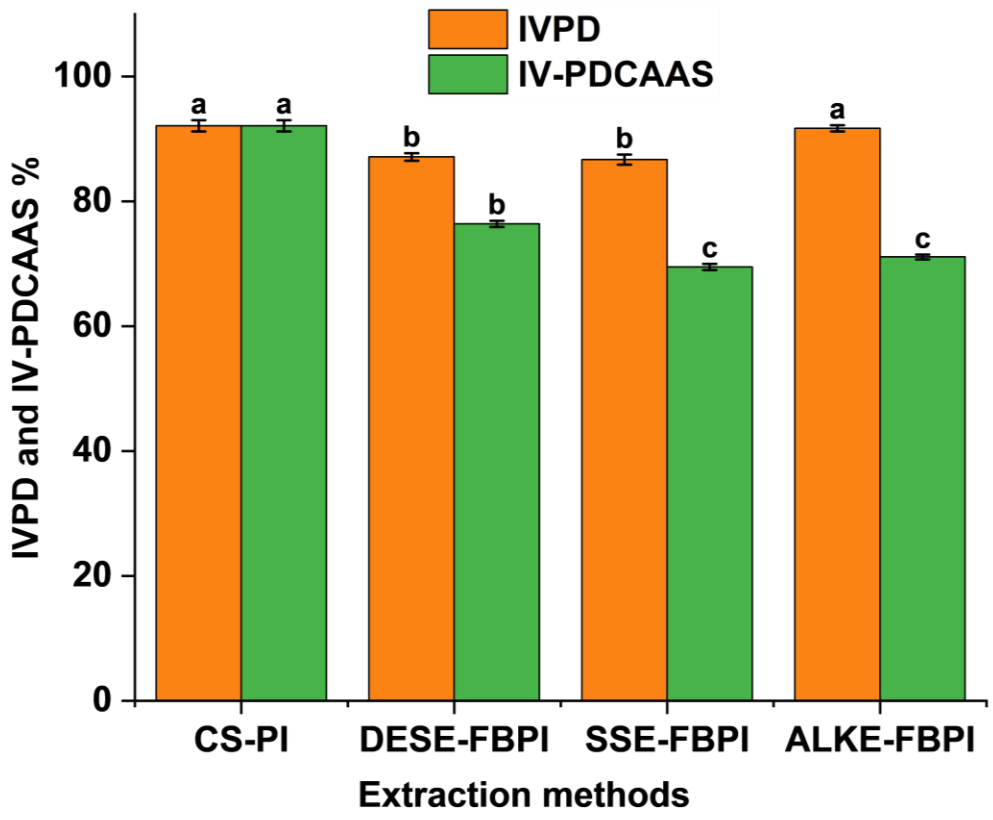

Figure 4. 8. *In vitro* protein digestibility (IVPD) and *in vitro* protein digestibility corrected amino acid score (IV-PDCAAS) of extracted protein isolates. DESE-FBPI, Deep eutectic solvent-extracted fava bean protein isolate; ALKE-FBPI, Alkaline-extracted fava bean protein isolate; SSE-FBPI, Salt-extracted fava bean protein isolate; and CS-PI, Commercial soybean protein isolate. All data were analyzed by analysis of variance followed by Tukey's range test (n=3). Different letters on the error bars bar graph indicate a significant difference ($P < 0.05$).

Proteins play a significant role in human nutrition. Nutritionally, the protein quality of a food is defined by its protein content, indispensable AAs profile, digestibility, and bioavailability (Sá et al., 2020). Although plant proteins can provide the required AA in the human diet (López et al., 2018), legume or cereal proteins are often regarded as incomplete proteins compared to animal proteins (Hertzler et al., 2020). In general, plant proteins have low digestibility due to the presence of antinutritional factors (Nosworthy et al., 2023), their rigid and compact protein structure, and the interaction of the protein with other compounds, such as carbohydrates, lipids, and polyphenols (Neji et al., 2022). However, the processing of legumes can significantly reduce these compounds and improve the protein quality. The AA composition and AA score of extracted protein isolates are summarized in Table 4.7, while IVPD and IV-PDCAAS values of the extracted FBPI are shown in Figure 4.8. The AA composition of DES-FBPI, ALKE-FBPI, and SSE-FBPI was comparable to established data (FAO/WHO, 1991), aligning with the reference amino acid scoring pattern, except for sulfur-containing AAs and tryptophan. Conversely, CS-PI showed an AA composition without any indispensable AA (IAA) deficiency. Regardless of the extraction method, the most abundant AA in all isolates, including CS-PI, were aspartic and glutamic acids, followed by arginine. Overall, ALKE-FBPI resulted in higher total AA content than all other methods, including CS-PI. All isolates obtained were rich in aromatic AAs, especially phenylalanine and tyrosine. Among them, CS-PI had the highest amount (86.5 mg/g), followed by DESE- FBPI (82.8 mg/g), ALKE-FBPI (81.0 mg/g), and SSE-FBPI (80.5 mg/g). The total IAA content in DES-extracted proteins was higher (426 mg/g) than that extracted using AE (419.9 mg/g) and SE (423.6 mg/g). A similar observation was reported by Lin et al. (2022) for DES-extracted seabuckthorn seed meal. These results confirm that DES-extracted proteins are a good source of AAs with higher nutritional quality, while the isolation technique determines the AA levels. The sulfur-containing AAs

(methionine and cysteine) were limiting in all the protein isolates, except for the CS-PI. However, it was the first limiting AA in proteins obtained from alkaline extraction, whereas tryptophan was the limiting AA in DESE-FBPI and SSE-FBPI. Thus, sulfur-containing AAs in DES and salt-extracted proteins were comparatively higher than those of ALKE-FBPI. Typically, the albumin fraction is rich in sulfur-containing AAs and is lost substantially during isoelectric precipitation. This was evident with the SDS-PAGE (Figure 4.3), where polypeptide bands corresponding to the albumin fraction almost disappeared in the ALKE-FBPI. Similar observations were reported by Jeganathan et al. (2023) and Vogelsang-O'Dwyer et al. (2020) regarding alkaline extraction.

Different protein extraction techniques can alter the protein's structural conformation through modification, thereby influencing its digestibility. The IVPD values differed significantly between the extraction methods ($P < 0.05$). The highest IVPD value was observed in the CS-PI and ALKE-FBPI. However, the other two extraction methods showed significantly lower values. The intense conditions involved during the alkaline extraction and industrial process could induce protein denaturation, exposing peptide bonds and increasing enzyme accessibility to susceptible sites (Drulyte & Orlien, 2019). This might be one of the reasons why both CS-PI and ALKE-FBPI showed higher IVPD values. Yu (2005) reported that a high content of secondary structure elements, β-structures, could limit access to proteolytic enzymes, thereby reducing protein digestibility. Carbonaro et al. (2012) demonstrated a strong negative correlation between the proportion of β-sheets and digestibility for common beans, chickpeas, lentils, soybeans, and barley. Additionally, they reported that intermolecular β-sheets were primarily associated with lower digestibility. Besides, several other factors can affect protein digestibility. The proportion of globular and albumin proteins in extracted isolates could contribute to changes in IVPD, where an increased albumin fraction has been reported to lower IVPD (Drulyte & Orlien, 2019). Even

though IVPD values of DESE-FBPI and SSE-FBPI were significantly lower than CS-PI and ALKE-FBPI, values were still above 85%, which is higher than some studies found in the literature for raw and processed fava bean proteins (64-78%) and similar to extruded fava beans (~87%) (Alonso et al., 2000; Khalil & Mansour, 1995; Sá et al., 2019). Furthermore, the digestibility value of protein isolates may also largely depend on the removal of anti-nutritional compounds during the extraction process. Potentially, the removal of anti-nutritional compounds in fava beans may be effective with an AE process followed by acid precipitation, as is supported by previous findings for fava beans (Vogelsang-O'Dwyer et al., 2020) and lentils (Joehnke et al., 2021). Interestingly, compared to IVPD, IV-PDCAAS exhibited an opposite trend with three extracted isolates. Like IVPD, CS-PI showed significantly higher IV-PDCAAS than the other three FBPI protein isolates. However, IV-PDCAAS of DESE-FBPI was found to be significantly higher compared to both ALKE-FBPI and SSE-FBPI. Nosworthy et al. (2018) reported the following *in vitro* PDCAAS for processed fava beans: extruded (54.5%), cooked (49.8%), and baked (57.5%), all of which are lower than the values obtained in this study. Furthermore, plant proteins, such as those found in potatoes, peas, and quinoa, have been reported to have a PDCAAS value of at least 75% (Hertzler et al., 2020). Therefore, fava bean protein obtained from DES extraction was claimed to be of higher quality. This would benefit food applications, such as blending with other plant proteins for improved nutritional properties.

Table 4. 7. Amino acid composition, amino acid score (AAS), *in vitro* protein digestibility (IVPD), and IV-PDCAAS of protein isolates.

AA composition (mg/g protein)	Requirement pattern[1] (mg/g protein)	CS-PI		DESE-FBPI		ALKE-FBPI		SSE-FBPI	
		AA	AAS[2] (%)	AA	AAS[2] (%)	AA	AAS[2] (%)	AA	AAS[2] (%)
Indispensable (IAA)									
Histidine (His)	19	28.4	149.3	28.8	151.3	28.3	149.1	29.5	155.3
Isoleucine (Ile)	28	46.7	166.8	46.8	167.0	46.9	167.5	46.3	165.3
Leucine (Leu)	66	78.9	119.6	81.0	122.7	83.3	126.2	79.6	120.6
Lysine (Lys)	58	63.5	109.5	68.0	117.3	66.9	115.4	69.8	120.4
Threonine (Thr)	34	36.7	108.0	36.8	108.3	35.0	102.9	37.0	108.9
Tryptophan (Trp)	11	13.9	126.6	9.6	87.7	9.4	85.4	8.8	80.1
Valine (Val)	35	47.5	135.7	49.8	142.2	49.7	142.0	48.9	139.9
Total sulfur amino acids (Met + Cys)	25	26.3	105.2	22.5	89.9	19.4	77.5	23.1	92.5
Total aromatic amino acids (Phe + Tyr)	63	86.5	137.3	82.8	131.4	81.0	128.5	80.5	127.7
Dispensable (DAA)									

Alanine (Ala)	-	43.1	-	43.0	-	41.6	-	43.2	-
Arginine (Arg)	-	71.0	-	87.1	-	89.8	-	86.4	-
Aspartic acid (Asp)	-	120.2	-	120.6	-	120.7	-	121.0	-
Glutamic acid (Glu)	-	198.5	-	184.8	-	189.8	-	186.6	-
Glycine (Gly)	-	39.2	-	41.0	-	40.4	-	41.3	-
Proline (Pro)	-	49.5	-	44.8	-	44.7	-	45.3	-
Serine (Ser)	-	50.5	-	52.5	-	53.0	-	52.6	-
Total IAA (mg/g protein)	-	428.5	-	426.0	-	419.9	-	423.6	-
Total DAA (mg/g protein)	-	571.5	-	574.0	-	580.1	-	576.4	-
Total AA (g/100g sample)	-	86.0	-	86.4	-	89.7	-	87.1	-
First limiting amino acid	-	No deficiency		Trp		Met+Cys		Trp	
Total hydrophobic AA		431.6		421.3		416.4		417.0	
Total hydrophilic AA		568.8		578.6		583.5		582.9	

[A–C] Different letters in the same row indicate a significant difference ($P < 0.05$ by Tukey's test).

[1] FAO/WHO (1991) Expert Consultation Report for amino acid scoring patterns for pre-school children (2-5 years old).

[2] AAS: Amino Acid Score = (mg of amino acid in 1 g of test protein/mg of amino acid in requirement pattern) × 100.

[3] IV–PDCAAS: *In Vitro* Protein Digestibility Corrected Amino Acid Score = AAS × IVPD.

CS-PI, Commercial soybean protein isolate; DESE-FBPI, Deep eutectic solvent-extracted fava bean protein isolate; ALKE-FBPI, Alkaline-extracted fava bean protein isolate; and SSE-FBPI, salt-extracted fava bean protein isolate.

4.6. Conclusion

Overall, this study investigated the effect of different extraction methods on the techno-functional properties and structural changes of fava bean protein isolates. Introducing novel fava bean protein ingredients with enhanced functionality to the global market is crucial. Thus, a comparison of the prepared fava bean proteins to the commercial protein ingredient, soybean protein isolate, was also investigated. The extraction technique significantly influenced the structure-functional properties of fava bean protein isolates. DES extraction was highly efficient in producing a protein isolate with a higher protein yield and protein content than conventional methods, suggesting that DES has potential for use in the industrial production of food proteins. In general, DES extraction exhibited better EAI and FC across different pH ranges studied, but showed poor functionality for FS and ESI compared to other samples. The relatively higher protein solubility, finer particle size distribution, and structural differences induced by DES extraction might have facilitated better functionality. However, among the protein isolates produced, ALKE-FBPI and CS-PI showed greater FS and ESI, respectively. Interestingly, among the FBPI, only DESE-FBPI could produce self-supporting firm gels similar to those of CS-PI, indicating potential innovation in fava bean protein gel-based food formulations. Hence, DESE-FBPI could be a viable alternative to replacing soybean-based food products, addressing the challenges posed by soybean protein allergenicity, which persists worldwide.

DESE-FBPI also had nutritional significance in terms of AA content and digestibility. ALKE-FBPI and CS-PI had higher IVPD than DESE-FBPI, probably due to the higher denaturation of proteins induced by the alkaline reagents. This indicates lower protein denaturation in DES extraction due to the mild extraction conditions employed. However, despite the lower IVPD of DESE-FBPI, the advantages of DES extraction are evident in improving protein quality,

as evidenced by higher IAA content and higher IV-PDCAAS compared to the SE and AE methods. Based on these results, DES extraction produced the best overall results in terms of both protein yield and functionality. Despite the higher nutritional quality of CS-PI compared to the other protein isolates, overall protein functionality was lower, possibly due to denaturation induced by the industrial isolation process. The major differences found between the fava bean protein prepared in the laboratory and commercially processed soybean proteins indicate that DES-extracted proteins produced in this study have favorable functionality for use in food products such as meat analogs, beverages, tofu, and yogurt. However, potential toxicity studies are still warranted for DES-extracted proteins to confirm their suitability in novel food applications.

Connection to Chapter 5

The functionalities of plant proteins play a crucial role in the development of plant-based foods, including meat analogs, gel products, beverages, emulsifiers, and bakery products. Combining high-quality plant proteins with plant-based products further enhances their nutritional value, which can mimic that of animal-based products. The fava bean protein extracted from DES showed promising potential in novel food applications, as revealed in Chapter 4, which evaluated its techno-functional properties and protein quality. The lower denaturation of DES-extracted fava bean protein resulted in better functionality, including solubility, emulsification, and foaming properties, than those obtained with conventional techniques. Interestingly, the gelation properties of DES extraction were remarkable, and it could produce firm, self-standing gels at a pH of 7 with a protein concentration of 20% w/v, similar to commercial soy protein isolates. Neither salt nor alkaline extraction could produce firm gels under these conditions. Moreover, DES extraction favored a greater legumin fraction in the isolates, resulting in a higher concentration of sulfur-containing amino acids, including essential amino acids, than those produced from other fava bean isolates via salt and alkaline extraction. This was reflected in the significantly improved protein digestibility-corrected amino acid score (>75%) of DES-extracted fava bean proteins, suggesting that DES could produce high-quality protein from fava beans for novel food applications. Hence, if there is potential for further improvement in protein yield, protein content, and protein functionality, it would benefit positioning the fava bean as a novel protein ingredient in the protein ingredient market to meet the ever-increasing demand for protein. Therefore, pre-treatment-assisted DES extraction would be one of the possible integrated approaches to achieve this target. Thus, Chapter 5 evaluates the effect of ultrasonication and enzymatic pretreatment-assisted DES extraction on fava bean protein yield and functionality. The pre-treatment methods, including

ultrasonication and enzymatic treatment, were optimized using the RSM model to achieve the optimal conditions. Protein extraction was then performed using the optimal extraction conditions obtained in Chapter 1. The protein yield, physicochemical, and functional properties were evaluated among pre-treated fava bean proteins and control (DES extraction without pre-treatment).

Chapter 5

Effect of Pre-treatment on Structural and Functional Properties of Deep Eutectic Solvent Extracted Fava Bean Protein Isolates:

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5.1. Abstract

In this study, two pre-treatment methods, ultrasonication (UP) and enzymatic hydrolysis (EP) using a multi-enzyme complex (Viscozyme L), were employed to isolate protein from fava beans using deep eutectic solvent (DES) extraction, compared to the control (without pre-treatment). The pre-treatment methods were optimized using the response surface methodology (RSM) to predict the best optimum variables for protein extraction yield. The protein yield obtained from UP and EP increased significantly by approximately 27% and 8%, respectively, compared to the control ($P<0.05$). The protein content of the control sample (DES-C) was 90.79 ± 1.64%, which was not significantly different from the protein extracted from the ultrasonication pre-treatment (DES-U). Nevertheless, protein from the EP (DES-E) had a higher purity (97.67 ± 0.26%) than both DES-C and DES-U ($P<0.05$). The volume-weighted mean particle size [D (4,3)] of the DES-U increased nearly 2-fold, while EP reduced D (4,3) approximately 2-fold compared to the control ($P < 0.05$). The UP and EP pre-treatments modified the secondary structural components by reducing the β-sheet and α-helix contents, significantly affecting the functional properties. Protein solubility, water, and oil holding capacity were significantly improved ($P < 0.05$) with the pre-treatments, but not the emulsifying and foaming properties. Gelling capacity, in particular, was significantly enhanced by the pre-treatments, resulting in the formation of firm gels within a broader pH range. The results suggest the potential for expanded use of fava bean protein isolates as a novel food ingredient in food formulations.

5.2. Introduction

With the growing demand for environmentally friendly and sustainable natural products, the food industry faces significant challenges in developing products bearing the “green” label. Especially, the demand for alternative proteins has grown tremendously due to their potential health benefits, minimal safety issues, and reduced potential for adverse environmental consequences when compared to animal proteins (Aschemann-Witzel et al., 2021). Despite the recent surge in the growing market segment for alternative plant protein products, developing protein ingredients and processing methods similar to their conventional counterparts remains challenging. Therefore, sustainable innovation in efficient green extraction and separation technologies is greatly in demand.

In recent years, deep eutectic solvents (DES), as a possible alternative to conventional solvents, have gained widespread acceptability amongst researchers in the field of protein extraction due to their unique physiochemical properties and sustainable characteristics (Hernández-Corroto et al., 2020; Hewage et al., 2022; Hewage et al., 2024; Liu et al., 2018; Yue et al., 2021). DES's unique hydrogen bonding interaction promotes the significant dissolution of intracellular components by breaking the resistant plant cell wall (Chen et al., 2023). In particular, DESs are considered designer solvents because their physiochemical parameters can be altered to tailor specific properties (Wazeer et al., 2018). For example, we demonstrated in one of our previous studies (Chapter 6) that varying proportions of hydration (H_2O) levels (0-100% w/w) in DES (Choline chloride/glycerol) significantly altered the protein extraction efficiency and modified secondary structure components in fava bean proteins, thereby affecting techno-functional properties. Therefore, DES may be designed to have unique solvent characteristics that favor specific food formulations. Although DESs exhibit superior performance compared to their

conventional counterparts, some bottlenecks, such as instability, high viscosity, and non-negligible vapor pressure, can impact the mass transfer rate (Gajardo-Parra et al., 2019). Despite the water substitutions in DES significantly improving the mass transfer rate (Hewage et al., 2024), the DES-water system may prodcue poorly soluble plant material due to a lower dispersion rate (Chen et al., 2023).

The plant cell wall is inherently rigid and dense, composed of cellulose, hemicellulose, pectin substances, and protein interwoven by various molecular forces. This rigid structure impedes solvents from penetrating cells and liberating cell constituents. It is worth noting that the DES method can extract approximately half of the available protein in fava beans, comparable to the alkaline method (Hewage et al., 2024). The other half possibly remains in the discarded insoluble materials. It is believed that the lower extractability of protein bodies is mainly due to their location in the plant cell and the formation of complexes with cellulose side chains of anarabinan and arabinogalactan (Kasai & Ikehara, 2005). Therefore, many enzymatic and physical treatment-assisted extractions increase protein extractability and subsequent protein modifications (Pojić et al., 2018). Among the physical treatments, ultrasonication (US) has garnered renewed attention in food processing research due to its energy efficiency, low maintenance costs, and reduced treatment time compared to other eco-innovative methods, such as high-pressure processing, pulsed electric fields, ultrasound, and microwave (Chemat et al., 2020). When the US is applied to a liquid medium, the cavitation effect creates violent microturbulence, generating a sudden burst of energy (Chemat et al., 2017; Rahman & Lamsal, 2021). This energy can effectively disintegrate the cell wall components, facilitating the transfer of proteins' increased diffusional mass into the extraction solvent. Currently, US-assisted protein extraction from fava beans using conventional methods has been reported in the literature, with promising increases in yield and

techno-functional properties (Alavi, Chen, & Emam-Djomeh, 2021; Badjona et al., 2024; Suchintita Das et al., 2023). Enzymatic pretreatment, on the other hand, is another tool used to improve protein extraction, considering its milder processing conditions. It requires a suitable cocktail of enzymes to rupture the cell wall. Hence, a combination of carbohydrases would facilitate the cell wall degradation, thereby liberating proteins into the solvent matrix (Liu et al., 2008; Rosset et al., 2014). For example, Viscozyme L is a multi-enzyme complex containing a wide range of carbohydrases, including arabanase, cellulase, beta-glucanase, hemicellulose, and xylanase, which can effectively hydrolyze plant cell wall polysaccharides (Novozymes, 2001). It has been demonstrated that promising improvements in plant protein extraction from oat bran (Liu et al., 2008), soybeans (Penha et al., 2020), and sesame bran (Görgüç et al., 2019) were achieved using Viscozyme L.

To our knowledge, DES-based enzymatic-assisted protein extraction has not been explored and is limited to US-assisted studies (Sharma et al., 2023). Therefore, this study aimed to provide a comprehensive approach to DES-based protein extraction from fava beans using US and enzyme-assisted methods. Initially, the protein was extracted using DES containing choline chloride /glycerol (1:2 molar ratio) according to our previous method (Hewage et al., 2024) as a control (DES-C). Both pre-treatment methods were optimized using the RSM statistical model with appropriate independent variables to predict the extraction yield. Following this, experimental validation was performed to extract the protein. The protein extracts were physiochemically characterized, followed by the evaluation of techno-functional properties compared to the control. Thus, the outcome of this study provides valuable insights into further investigation and the potential industrial scaling up of DES-based novel technologies-assisted protein extraction.

5.3. Materials and Methods

5.3.1. Materials

Snowbird fava bean seeds (dehulled) from a non-tannin variety were purchased from Prairie Fava, MB, Canada. Laemmli sample buffer, 2-mercaptoethanol, Precision Plus protein standard (10-250 kDa), Coomassie Blue R-250, and 4-15% Mini-PROTEAN TGX precast protein gels were purchased from Bio-Rad Laboratories Ltd. (Mississauga, ON, Canada). Sunflower oil was purchased from a local shop in Winnipeg, Canada. Viscozyme enzyme was purchased from MilliporeSigma Canada Ltd (Oakville, ON, Canada). All analytical-grade chemicals were purchased from Fisher Scientific (Ottawa, ON, Canada). Fava bean seeds were milled using Prater-Sterling Impact Mill (M-21, Prater Industries, Bolingbrook, IL, USA.) equipped with a 0.2 mm screen and stored at -20 °C until further analysis.

5.3.2. Methodology

5.3.2.1. Protein extraction using DES

DES protein extraction was performed using optimized conditions as previously reported (Hewage et al., 2024) with minor modifications. Briefly, fava bean flour (FBF) was mixed with 60% (w/w) DES containing choline chloride and Glycerol (1:2 molar ratio, w/w) and 40% water (w/w) at a 12.5:1 liquid-to-solid ratio. The remaining procedure was similar to our previously reported method (Hewage et al., 2024). The protein isolate obtained was referred to as DES-control (DES-C).

5.3.2.2. Optimization of ultrasonic and enzymatic pre-treatment

5.3.2.2.1. Ultrasonic pre-treatment (UP)

Optimization was done to determine the optimum parameters for achieving the highest protein extraction yield. A full fraction central composite design (CCD) under the RSM statistical model was used to optimize the two independent variables, amplitude% and time (min). These variables were tested at four distinct levels, as presented in Table 5.1. The appropriate ranges of independent variables were chosen according to the previously reported data (Tang et al., 2010). The CCD generated 13 runs with five center points (Table 5.2). The response variable, extraction yield, was then fitted to a second-order polynomial model (Eq.1) to evaluate its adequacy. Finally, the extraction yield was experimentally validated using the optimum conditions obtained from the RSM model.

$$Y = \beta_0 + \sum_{i=1}^{k} \beta_i x_i + \sum_{i=1}^{k} \beta_{ii} x_i^2 + \sum_{i<j}^{k} \beta_{ij} x_i x_j + \varepsilon \quad (1)$$

where Y is the predicted response variable for protein yield (%); β0 is the regression coefficient (constant term); βi, βii, and βij are the coefficients for the linear, quadratic, and interaction effects, respectively.

RSM was performed using the MINITAB software (MINITAB version 20, LLC, PA, USA), and analysis of variance (ANOVA) was used to determine the coefficient of determination (R^2) and the P values of the lack-of-fit tests at a significance level of $P > 0.05$. The 3D Surface plot was generated in Statistica (v. 13.5, StatSoft Inc., Tulsa, USA) to visualize the interactive effects of the variables.

Table 5. 1. Independent variables used in experimental design (CCD) for UP-assisted DES protein extraction

Input Variables	Levels				
	-1.41	-1	0	+1	+1.41
Amplitude % (X_1)	40	46	60	74	80
Time (min) ($X2$)	2	4	8.5	13	15

Central composite design (CCD) in Response Surface Methodology (RSM)

5.3.2.3. Protein extraction using ultrasonic pre-treatment (UP)

FBF dispersion with DES was prepared according to the method outlined in section 5.3.2.1. The ultrasonication treatment conditions, generated based on the RSM design matrix, as illustrated in Table 5.2, were achieved using a sonicator equipped with a 20 kHz frequency and 500 W power output (Fisher Scientific, Pittsburgh, PA, USA). First, the sample was placed in an ice water bath to control the temperature of the solvent medium, which was attained through sonication. Then, the sonicator probe (10 mm diameter) was immersed in the flour dispersion, and protein extraction was performed according to the previously described optimized procedure (Section 5.3.2.1). The protein isolate obtained using the UP method is herein referred to as DES-U.

5.3.2.3.1. Enzymatic pre-treatment (EP)

As a preliminary study, the DES-based enzyme-assisted DES extraction was optimized using the same statistical model and the design employed in the UP method (section 5.3.2.2.1). Three independent variables, enzyme concentration (Fungal Beta-Glucanase units FBG /10 g of substrate), pH, and temperature (°C) with appropriate ranges, were determined according to the product sheet of Viscozyme L (Viscozyme® L. Product Sheet. 2001) and from the previously

reported data (Liu et al., 2008). The CCD design generated 20 runs with six center points at five levels, as indicated in Table 5.3S (Appendix 8). Enzyme-assisted DES protein extraction was performed using the procedure outlined in Section 5.3.2.1, with adjustments made to the enzymatic conditions accordingly. In brief, 10 g of fava bean flour was mixed with 125 mL of DES at a 1:12.5 (w/w) solid-to-liquid ratio and homogenized. Then, the pH (3.0–5.0) and Viscozyme L concentration (6–30 FBG/10 g of substrate) were adjusted according to the design matrix (Table 5.4S in Appendix 9). The enzymatic treatment was carried out at 200 rpm using the same circulation water bath for 1 hrs at different temperatures (30–50 °C). After the enzymatic pre-treatment, protein extraction was further continued for 1 hrs based on the previously described method (section 5.3.2.1). However, the optimization study results indicated a lower protein yield (Table 5.6S**,** Appendix 11) than that of DES-C. Therefore, optimum enzymatic conditions predicted from this preliminary study were used to modify the enzyme-assisted DES extraction process.

5.3.2.3.2. Protein extraction using the modified enzymatic pre-treatment (EP)

Ten grams of fava bean flour was dispersed in 50 g of deionized water and homogenized (Model 850, Fisher Scientific, Ottawa, ON, Canada) at 20,000 rpm for 1 min. The resulting slurry was stirred for 1 hrs at an ambient temperature. Afterward, the slurry was adjusted to enzyme conditions obtained from the preliminary study, i.e., pH 5.0, temperature 38.5 °C, and enzyme concentration 30 FBG/10 g of substrate. Pre-treatment was allowed for 1, 2, and 3 hrs separately as a single-factor experiment. Soon after the enzymatic reaction, the mixture was adjusted to pH 7.0, and the appropriate amount of DES (75 g) added (solid: liquid ratio, 1:12.5) while maintaining the pH at 7.0. Then, the slurry was stirred for 15 min, followed by protein extraction as described in section 5.3.2.1. Immediately after extraction, the sample was cooled in an ice bath, centrifuged

(17,709 g, 4 °C for 20 min), dialyzed, and freeze-dried to obtain the protein isolate, which was named DES-E.

5.3.3. Proximate analysis

Protein samples were weighed (5 g) and dried in an oven (Thermo Fisher Scientific, Pittsburgh, PA, USA) at 105°C until a constant weight was achieved to determine the moisture content. The protein content was determined by the Kjeldahl method, using the conversion factor of 6.25 (Feng et al., 2025). The ash content was analyzed using a muffle furnace at 550°C overnight (Model 1100 box furnace, Thermo Scientific, NC, USA), and the crude fat content was determined using the Soxhlet method (AOAC, 2010). The total carbohydrate content was calculated using the difference method, where the total equals 100 minus the sum of protein, fat, ash, and moisture.

5.3.4. Characterization of isolated protein fractions

5.3.4.1. Color

The color profile of extracted protein isolates was characterized using a color spectrophotometer (RM 200, Lovibond LC 100, Tintometer Group, China). The sample reflectance was measured based on the L*, a*, and b* values on the Hunter scale.

5.3.4.2. Particle size distribution

The particle size distribution of pre-treated and control protein dispersions was measured using light scattering with a Mastersizer 3000, equipped with a hydro dispersion unit from Malvern Instruments Ltd. (UK). Each freeze dried protein sample, at a concentration of 1% (w/v), was thoroughly mixed overnight at 4 °C in 0.1 M phosphate buffer, pH 7.0. Then, the samples were equilibrated at room temperature (23°C) for 5 hrs. Particle distribution was analyzed by transferring the samples into the liquid dispersion unit, using laser obscuration, with the following parameters: a particle refractive index of ~12%, absorption index of 1.45, dispersant refractive

index of 0.1, and 1.33, respectively. The results were reported as the volume-weighted mean particle diameter (D 4,3), surface-area-weighted mean particle diameter (D 3,2), and 50th percentile [D (0.5)] (Arzeni et al., 2012), with averages and standard deviations calculated from three readings made on two replicates.

5.3.4.3. Surface hydrophobicity (S_o)

S_o was determined using 1-anilino-8-naphthalenesulfonate (ANS) as a fluorescence probe, according to the method of Oluwajuyitan & Aluko (2024) with slight modifications. The pre-treated and control samples were dispersed into a 0.1 M sodium phosphate buffer solution (pH 7.0), stirred for 2 hrs at ambient temperature, and then centrifuged at 10,000 g for 10 min at 23 °C. After determining the protein content of the resulting supernatants, each supernatant was prepared into a 50-250 µg/mL serial dilution. Then, 20 µL of ANS solution (0.8 M in the same phosphate buffer) was added to 250 µL of each sample. Fluorescence intensity was measured using a spectrofluoriemeter (Jasco-FP-6300, Tokyo, Japan) at excitation and emission wavelengths of 390 nm and 470 nm, respectively. The fluorescence intensity versus protein concentration (mg/mL) was analyzed using a linear regression model, and the slope taken as the S_o.

5.3.4.4. Attenuated total reflectance Fourier-transformed infrared spectroscopy (ATR-FTIR)

The FTIR spectroscopy of the extracted protein samples was obtained from an ATR-FTIR spectrometer (INVENIO S, Bruker Scientific LLC., MA, USA). The spectral data were collected in the 400–4000 cm^{-1} range with an average of 120 scans and a resolution of 4 cm^{-1}. The spectral data were normalized, and then baseline reduction was performed to obtain the second derivative curve. The peak fitting in the amide-I (1700–1600 cm^{-1}) region was evaluated after applying the Savitzky-Golay smoothing function using Origin 2024 software (OriginLab Corporation, MA, USA) to quantify the secondary structure components.

5.3.4.5. Sodium dodecyl sulfate-polyacrylamide gel electrophorosis

SDS-PAGE was run to determine pre-treatment effects on the protein polypeptide profiles under reducing and non-reducing conditions according to the method reported by Flores-Jiménez et al. (2019) with alterations. Briefly, protein samples were dissolved in 5% SDS (w/v) and heated at 85 °C for 1 hrs followed by centrifugation (10,000 g, 15 min, 23 °C). Protein concentration of the supernatant was measured using the Bradford method. Thereafter, each protein sample (4 mg/mL) was mixed with an equal volume of Laemmli buffer that contained (reducing condition) or did not contain (non-reducing condition) mercaptoethanol to give a final protein concentration of 2 mg/mL. These samples were heated at 90 °C for 5 min, and 5 µL of supernatant and 10 µL of Precision Plus protein standard (Bio-Rad Laboratories Inc., CA, USA) were loaded onto the gel lanes, which comprised 4-15% Mini-Protean TGX precast gels. (Mini-Protean II electrophoresis unit, Bio-Rad Laboratories Inc., CA, USA). After electrophoresis (150 V), the gels were stained with Coomassie blue (R-250) for 1 hrs followed by destaining (2 hrs) with solvent solution containing 50% (v/v) methanol and 10% (v/v) acetic acid in water.

5.3.4.6. Thermal stability

Differential scanning calorimetry (DSC) (Model DSC2500, TA Instruments-Waters LLC, DE, USA) was used to determine the onset temperature (T_o), peak denaturation temperature (T_d), and enthalpy (ΔH) for protein samples that were pre-dried in a desiccator containing P_2O_5 for 7 days. The thermal parameters were determined by heating approximately 5 mg of each sample in hermetically sealed aluminum pans from 30 to 250 °C at 10 °C/min, after equilibration at 30 °C, with an empty pan used as a reference. The thermodynamic data were calculated using TA Universal Analysis 2000 software (Model DSC2500, TA Instruments-Waters LLC, DE, USA) and reported as the average value of at least two replicates.

5.3.4.7. Surface morphology

The surface morphology of the extracted proteins was analyzed using scanning electron microscopy (SEM) (Quanta 650 FEG, FEI Company, OR, USA). A thin layer of freeze-dried protein samples was carefully placed on the conductive adhesive carbon tape attached to the sample stub. Then, the sample was sputter-coated (Denton Vacuum Desk II, Denton Vacuum Inc., NJ, USA) with gold-palladium (Au-Pd) to enhance visualization of its microscopic structure. The microscopic images were analyzed using xT microscope control software (ver. 6.2.8) (Quanta 650 FEG, FEI Company, OR, USA) at various magnification factors using 5 kV voltage.

5.3.5. Evaluation of functional properties

5.3.5.1. Solubility

Protein solubility was measured according to the method outlined by Li et al. (2020). Approximately 0.1 g of samples were stirred with 20 mL of Milli-Q water overnight at 4 °C to thoroughly disperse the proteins. Then, the protein solutions were adjusted to pH 3.0, 5.0, 7.0, 9.0, and 11.0 using 1 M NaOH or 1 M HCl. The solutions were stirred (200 rpm, 1 hrs, 23 °C) followed by centrifugation (10,000 g, 20 min, 4 °C). The protein content in the supernatant was determined by the Kjeldahl method, and solubility (%) was calculated as per g of soluble protein in the supernatant per g of total protein in the initial sample.

5.3.5.2. Water holding capacity (WHC) and Oil holding capacity (OHC)

The Oil-holding capacity was determined using the procedure described by Malomo et al. (2014) with slight modifications. Samples (1 g) were mixed with 20 mL of sunflower oil in a pre-weighed Falcon tube for 2 min. After centrifugation at 4000 g for 30 min at 23 °C, the supernatant was discarded, and the tubes were inverted to drain the excess water for 15 min. The tubes were then dried at 50 °C in the oven for 30 min and reweighed. OHC was measured using the same

method as WHC, except for oven drying, by dispersing a 1 g protein sample in 10 mL of sunflower oil. The percentages of WHC and OHC were calculated using Equation (2).

$$WHC\ or\ OHC\% = \frac{Weight\ of\ the\ water\ or\ oil\ absorbed\ by\ sample}{Weight\ of\ the\ initial\ sample} \times 100 \quad (2)$$

5.3.5.3. Emulsification activity index (EAI) and stability index (ESI)

The emulsion activity index (EAI) and stability index (ESI) of the samples at pH 7.0 were determined using a few alterations to the turbidimetric method described by Zhang et al. (2021). Ten milliliters of sample (1% w/v protein concentration) was first prepared in 0.1 M phosphate buffer (pH 7.0) and stirred at room temperature (23 °C) for 1 hrs. Subsequently, 5 mL of sunflower oil was mixed and mechanically homogenized at 20,000 rpm for 1 min using a 10 mm sawtooth probe (Model 850, Fisher Scientific, Ottawa, ON, Canada). Afterward, 50 µL of the newly formed emulsion was immediately withdrawn from the bottom of the tube and dispersed in 5 mL of 0.1% (w/v) SDS solution. Then, the absorbance of the emulsion was measured at 500 nm using a UV-spectrophotometer (Genesys 150, Madison, USA) at 0 min (A0) and 10 min (A_{10}). EAI and ESI were calculated using Equations (2) and (3).

$$EAI\ (m^2/g) = \frac{2 \times 2.303 \times A_0 \times N}{c \times \varphi \times 10000} \quad (3)$$

$$ESI\ (\text{min}) = \frac{A_0}{\Delta A} \times t \quad (4)$$

Where A_0 and A_{10} were the absorbances of diluted emulsion at 0 and 10 min after homogenization, respectively, C was the weight of protein per volume (g/mL), D was the dilution factor (100), ϕ

was the volume fraction of sunflower oil (0.25), ΔA is the change in absorbance between 0 and 10 min (A_0–A_{10}), and t is the time interval, 10 min.

5.3.5.4. Foaming capacity (FC) and stability (FS)

To determine the foaming properties, the following method was adopted with modifications (Amiri et al., 2018). Briefly, 20 mL of 1% (w/v) samples prepared in 0.1 M phosphate buffer (pH 7.0) were stirred for 1 hrs at room temperature (23 °C). Foams were generated by a homogenizer at 11,000 rpm for 2 min (Model 850, Fisher Scientific, Ottawa, ON, Canada) with a 20 mm shaft. Afterward, the initial foam volume was recorded (V_0) within 10 s and then allowed to stand for 30 min at room temperature (23 °C). The foam volume was then measured (V_1), and FC and FS were calculated using Equations (5) and (6).

$$FC\% = \left(\frac{V_0}{20mL}\right) \times 100 \quad (5)$$

$$FS\% = \left(\frac{V_1}{V_0}\right) \times 100 \quad (6)$$

5.3.5.5. Least gel concentration (LGC)

LGC was determined according to the method outlined by Benelhadj et al. (2016) with some alterations. Samples were mixed with 5 mL of distilled water in a 15 mL Falcon tube to obtain 5, 8, 10, 12, 15, and 20% (w/v) protein concentrations, followed by stirring at 200 rpm for 2 hrs. Then, protein dispersions were adjusted to pH 3.0, 5.0, 7.0, and 9.0 using 0.1 M NaOH or HCl, followed by heating at 90 °C for 1 hrs in a water bath. The gel formed by Falcon tubes was immediately cooled using running tap water and stored overnight at 4 °C. Subsequently, the LGC was determined as the concentration at which the gel did not slip out when the sample tubes were inverted.

5.3.6. Statistical analysis

Unless otherwise stated, all the tests were conducted in triplicate, and the results are given as the means ± standard deviations. The results were analyzed using one-way analysis of variance (ANOVA), and the Tukey test was performed to determine significant differences between samples within the 95% confidence interval ($P < 0.05$) using MINITAB statistical software (MINITAB version 20, LLC, PA, USA).

5.4. Results and Discussion

5.4.1. Optimization of ultrasonication pre-treatment

The results for optimizing ultrasound pre-treatment assisted (UP) DES extraction are shown in Table 5.2. Under the experimental conditions, the protein yield varied between 63% and 68.90%. The highest protein yield (68.86%) was achieved with a 60% amplitude and a duration of 7.5 min. A positive correlation was observed between the protein yield and the amplitude over time, Figure 5.1(a). As the amplitude increases, cavitation bubbles become more violent and distort the plant cell wall, increasing solvent penetration and extraction yield (Shen et al., 2022). However, the lowest protein yield was recorded as 63.72% when the amplitude was applied at 74% for 13 min. This implies that the longer the duration of ultrasonication with increased power, the lower the protein yield. The cavitation effects of the sonication and the resulting microturbulence in the extraction medium may negatively impact proteins, leading to denaturation and aggregation, thereby affecting solubility (Rahman & Lamsal, 2021). Reactive species such as OH• and H• can also be generated as cavitation breaks down the water molecule. These reactive species can be detrimental to the protein, initiating protein oxidation, which could induce protein aggregation and change the protein's conformation (Rahman et al., 2020).

The optimization was achieved using RSM to develop the prediction model. The independent and dependent variables were analyzed for a second-order polynomial regression equation to predict the response variable (yield) within the adopted experimental conditions. The regression equation for the predicted protein yield (Y) is presented in equation (7).

$$\text{Protein Yield } (Y\%) = 47.96 + 0.415\,X1 + 2.291\,X2 - 0.00278\,X1*X1 - 0.1167\,X2*X2 - 0.01054\,X1*X2 \quad (7)$$

The results of the Analysis of Variance (ANOVA) for the CCD are presented in Table 5.2S (Appendix 7). Based on the results, the regression model was statistically significant ($P < 0.001$), indicating that the generated polynomial model accurately represents the relationship between the response variable and the chosen parameters. The coefficient of variation (R^2) and the lack of fit of the model were 0.9636 and 0.092, respectively. This suggested that the model provided a good fit for predicting protein yield under the experimental conditions used in this study.

Table 5.2. Central composite design (CCD) matrix and response variable (protein yield%) predicted from UP-assisted DES protein extraction

Run	Independent variables [a]		Protein yield % (DW) [b]
	Amplitude % (X1)	Time (min) (X2)	
1	60 (0)	8.5 (0)	68.78
2	74 (+1)	13 (+1)	63.72
3	80 (+1.41)	8.5 (0)	66.85
4	60 (0)	2 (-1.41)	66.20
5	40 (-1.41)	8.5 (0)	68.27

6	60 (0)	8.5 (0)	68.76
7	60 (0)	15 (+1.41)	61.28
8	60 (0)	8.5 (0)	68.86
9	46 (-1)	4 (-1)	65.68
10	60 (0)	8.5 (0)	68.02
11	74 (+1)	4 (-1)	67.61
12	60 (0)	8.5 (0)	68.28
13	46 (-1)	13 (+1)	64.53

[a] Coded symbols and levels of independent variables refer to Table 1.

[b] Averages of duplicated determination predicted protein yield % on a dry weight basis (DW)

The interaction effect of UP conditions on fava bean DES protein extraction, fitted by a second-order polynomial equation, is presented in Table 5.2S (Appendix 7) and Figure 5.1(b). In this model, linear terms, except for the amplitude percentage and all square terms, were significant ($P < 0.05$), whereas the interaction term (Amplitude × Time) was not significant ($P > 0.05$). The predicted protein yield variations within the selected treatment combinations were insignificant (Table 5.2). Thus, the interaction effect between the treatment parameters could not be observed. When increasing both the amplitude (40-62%) and time (2-7 min), the yield increased to its maximum and decreased as further treatment progressed. Interestingly, the reduction in protein yield was drastic when the treatment time was increased, rather than the amplitude [Figure 5.1(b)]. Hence, treatment time was considered a major factor in the UP-assisted DES protein extraction. This was further evidenced by the significant ($P < 0.05$) linear and quadratic effects of treatment

time on the response. Therefore, the optimum conditions, i.e., an amplitude of 62% and a time of 7 min, were chosen for pre-treatment in DES protein extraction from fava beans.

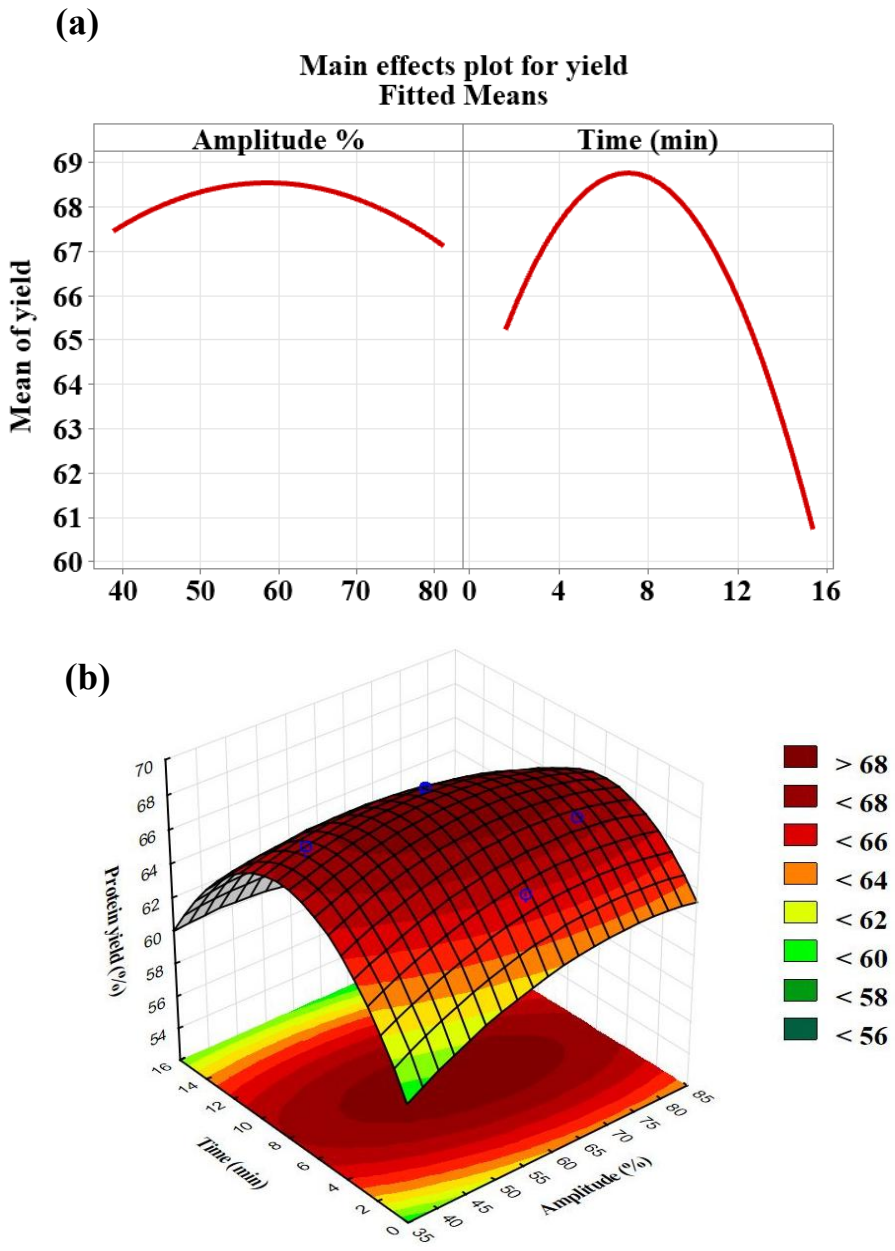

Figure 5. 1(a) Main effects plots for the yield of fitted means by amplitude (%) and time (min), and response surface plots for the interaction effects, **(b)** in ultrasound-assisted DES protein

extraction from fava beans. Interaction terms obtained from the polynomial equation of the RSM model with $P < 0.05$ and lack of fit $P > 0.05$.

Table 5.1S (Appendix 6) presents the optimum pre-treatment conditions derived from the regression model and their corresponding validation results. Under optimum conditions, with an amplitude of 62% and a treatment time of 7 min, the maximum predicted protein yield was 68.77%. The model was validated experimentally using the optimum treatment conditions predicted from the model. The results indicated that the experimental value for protein yield (68.48 ± 1.38%) was close to the predicted value (68.77%). Therefore, the generated second-order polynomial model was suitable for predicting the protein yield by UP-assisted DES extraction.

5.4.2. Optimization of enzymatic pre-treatment

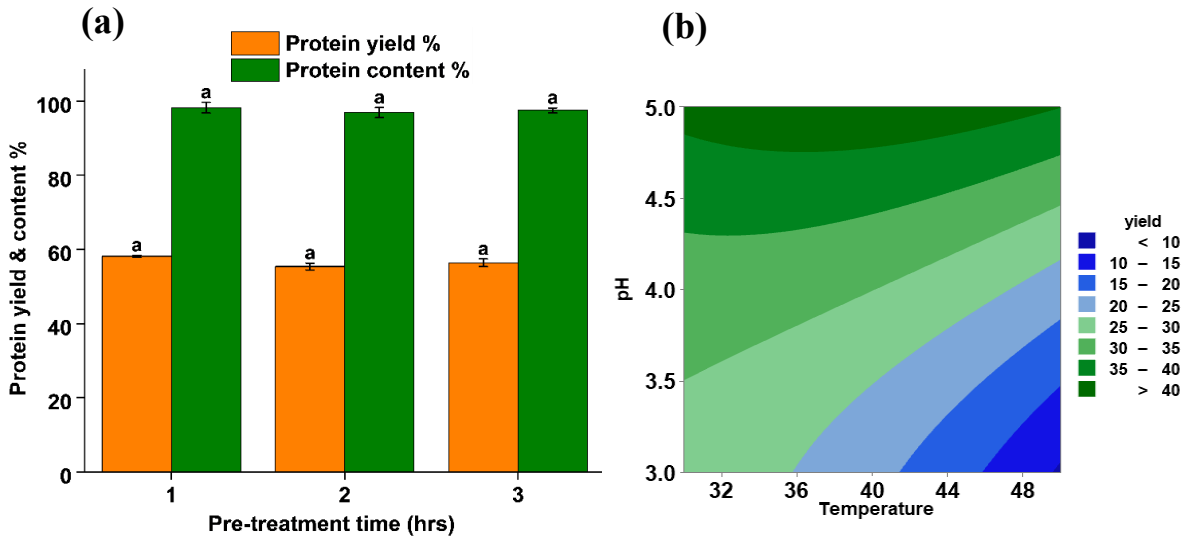

Figure 5. 2 (a). Protein yield and protein content obtained under the optimized enzymatic pre-treatment conditions at three different time slots (1-3 hrs); enzyme concentration 30 FBG/10g substrate, temperature 38.5 °C, pH 5.0., **(b)** contour plot for the interaction effect of temperature and pH versus protein yield under the RSM optimization of enzymatic pre-treatment. All results

are presented as the mean ± standard deviation (n = 3) on a dry weight basis. Different superscripts within a column indicate significant differences ($P < 0.05$).

The preliminary study was conducted to optimize enzymatic pre-treatment using the RSM-generated model. The optimized protein yield results (Table 5.4S-Appendix 9), ANOVA analysis (Table 5.5S-Appendix 10), and the optimum enzymatic conditions (Table 5.6S-Appendix 11) achieved are presented in the supplementary information section. Based on the probability value ($P < 0.001$), R^2 (0.9577), and the lack of fit ($P > 0.05$) of the regression model, it can be suggested that the data are well-fitted to predict protein yield within the chosen parameters. The impact of pH and temperature on enzymatic activity was substantial for predicting protein yield, as their interaction was significant ($P < 0.05$) [Figure 5.2(b)]. The optimized enzymatic pre-treatment conditions were as follows: enzyme concentration, 30 FBG/10g substrate; temperature, 38.5 °C; and pH, 5.0. Under these conditions, the predicted and the validated protein yields were 43.76% and 45.36%, respectively. The optimum pH range and temperature of Viscozyme L was reported as 3.3–5.5 and 25-55 °C, respectively, on the manufacturing sheet prepared by Novozymes. The current results showed that the temperature and the pH values are consistent with the reported values by Novozyme and those obtained for the oat bran (Liu et al., 2008). The protein yield obtained in this study is comparatively lower than that of the control (DES-C) sample (53.74 ± 0.19%). The optimum pH activity range of Viscozyme L is close to the isoelectric point of the fava bean proteins (Kamani et al., 2024). Therefore, the extracted proteins precipitated during the pre-treatment, which affected the protein yield. Although a subsequent adjustment to pH 7.0 was made for the DES extraction, the precipitated proteins did not completely dissolve in the DES system. Moreover, the DES system consisted of 40% water by weight, providing the medium for enzyme hydrolysis to degrade the cell wall. Nevertheless, 60% w/w DES still made the system

slightly viscous, which could impede the penetration of enzymes to the active site of the cell wall, thereby reducing the catalytic activity. However, it is interesting that optimization achieved the highest enzyme concentration (30 FBG/10g) among the chosen range. This means that when the enzyme concentration increases, protein yield also increases (linear term, $P < 0.05$), indicating that the carbohydrase's catalytic activity can be retained in the DES system. Due to these reasons, enzymatic pre-treatment was performed entirely in aqueous media (water) using the optimized conditions obtained from the preliminary study. Then, the subsequent extraction was carried out by adding the appropriate amount of DES, along with the pH adjustment. The protein yield resulting from the three different time slots under optimized conditions is shown in Figure 5.2(a). No significant difference was found ($P > 0.05$) in protein yield over the tested time, and the values obtained were higher than those of DES-C. These results showed that the optimized enzymatic pre-treatment conditions of enzyme concentration (30 FBG/10g substrate), temperature (38.5 °C), pH 5.0, and time (1 hrs), could yield 58.14% protein.

5.4.3. Protein yield and proximate composition of protein isolates obtained from pre-treatment-assisted DES protein extraction

The protein yield and proximate composition of fava bean protein isolates extracted using various pre-treatment methods are given in Table 5.3. The extraction yield of the control sample was 53.74 ± 0.19%, which was significantly enhanced ($P<0.05$) after UP (68.48 ± 1.82%) and EP (58.14 ± 0.27%). A similar observation for conventional extraction methods was previously reported by other researchers (Byanju et al., 2020; Turker & Isleroglu, 2024; Wang et al., 2021; Yang et al., 2024). As can be seen, among the pre-treatments, UP yielded a significantly higher protein content ($P<0.05$) than EP. Compared to the control, the UP yielded approximately a 27% increase, whereas the EP improved the yield by only about 8%. The robust cell wall destruction

exerted by the UP within a short period could result in higher cellular permeability, thereby enhancing protein release into the extraction solvent. This can significantly improve the protein extraction yield compared to the control. However, the other cell constituents, such as starch, may increase with the UP and contribute to the total protein yield, potentially affecting protein purity, which aligns with the results presented in Table 5.3. On the other hand, the EP may break down the cell wall's complex carbohydrates more effectively at a slower rate, releasing almost all of the protein bodies in the cell matrix. That is possibly why EP produced significantly higher protein content (97.67 ± 0.26%) than the DES-U (88.78 ± 0.94%). The current results agree with those of Byanju et al. (2020), who reported that the decreased protein purity of soybean, kidney bean, and chickpea proteins after ultrasonication was applied was due to the presence of other compounds, such as oils, sugars, and isoflavones. Latif & Anwar (2011) also made a similar observation for the Viscozyme L-treated sesame seed proteins, similar to those obtained through solvent extraction. The high purity observed for the DES-E can be attributed to the optimum conditions obtained from the RSM model, which effectively hydrolyzes cell wall interferences in the aqueous medium, thereby liberating more proteins for solubilization in the DES. Notably, it can be inferred that the protein liberated from the EP interacted well with the DES components, thus resulting in greater solubility.

Regarding the proximate composition of the pre-treated fava bean protein isolates (FBPI), the moisture content varied significantly across the samples, ranging from 8.07 ± 0.09% to 10.36 ± 0.79%. Both DES-U and DES-E had lower moisture contents ($P<0.05$) than the DES-C. When the sample was exposed to ultrasound, the resulting alternate compression and rarefaction could increase the acoustic dehydration (Awad et al., 2012). The ash content of all samples was below ~3%. It was found that DES-U had a significantly lower ash contents (1.52 ± 0.07 %) than both

DES-C (2.78 ± 0.63%) and DES-E (2.53 ± 0.30%), which did not show a significant difference between each other. The enzymatic hydrolysis of the cell wall can release electrostatically and covalently bound minerals, such as Ca and Mg, from pectin (Rose, 2003). However, DES has been shown to offer greater capabilities in removing lignocellulosic materials from biomass (Li et al., 2023; Zhang et al., 2016), during which some minerals may contribute to the extracted proteins. The significantly lower ash content in DES-U (1.52 ± 0.07) may be attributed to the washout of cell wall constituents with bound minerals following disruption by UP. Interestingly, the fat content of DES-E was in trace amounts, whereas both DES-C and DES-U contained 1.77 ± 0.35% and 2.31 ± 1.13% fat, respectively, and these differences were not significant ($P > 0.05$).

Table 5.3. Protein yield and proximate composition of protein isolates extracted from various pre-treatment methods

Pre-treatment method	Protein yield %	Moisture %	Ash %	Fat %	Protein %	Total CHO *%
DES-C	53.74±0.19^{c}	10.36±0.79^{a}	2.78±0.63^{a}	1.77±0.35^{a}	90.79±1.64^{b}	4.66
DES-U	68.48±1.82^{a}	7.92±0.91^{b}	1.52±0.07^{b}	2.31±1.13^{a}	88.78±0.94^{b}	7.39
DES-E	58.14±0.27^{b}	8.07±0.09^{b}	2.53±0.30^{a}	ND	97.67±0.26^{a}	ND

Fava bean protein isolates were extracted from various pre-treatment-assisted DES extraction methods, *i.e.*, Control sample (DES-C), Ultrasound-assisted fava bean protein isolates (DES-U), Enzyme-assisted fava bean protein isolates (DES-E). Results are presented as mean ± standard deviation (n=3) on a dry weight basis. Different superscripts within a column indicate significant differences ($P < 0.05$). * Calculated by differential from 100%. ND- Not Detected (trace amount).

5.4.4. Physiochemical characterization of protein isolates obtained from pre-treatment-assisted DES protein extraction

5.4.4.1. Changes in protein Color

Color is an important sensory attribute for product acceptability and marketability among consumers. The effect of UP and EP on the color of the protein isolates compared to the control is presented in Table 5.4. and (Figure 5.1S in Appendix 12). The *L** value (lightness) significantly decreased for both pre-treated samples compared to DES-C. However, no significant difference was found with the *a**(red/green coordinates*)* for all cases. Similar to *L** values, the *b** values (yellow/blue coordinates) also showed significant differences between each protein, ranging from 10.07 ± 0.15 to 16.30 ± 0.36, with the following order of decreasing values: DES-E > DES-C > DES-U. The effect of ultrasound depends on the treatment conditions employed and the intrinsic characteristics of the food material (Bi et al., 2015). The pigment present in the fava bean flour, such as anthocyanins, carotenoids, ascorbic, and phenolic compounds, could be affected by the sonication, especially with the energy exerted through the turbulence mechanism, and also pyrolysis and oxidation by free radicals formed by cavitation (Pingret et al., 2013). Thus, this can provoke substantial quality defects in food products. In addition, erosion, shear forces, fragmentation, and capillary effect, resulting in gradual physical damage to plant tissue, could be associated with color changes (Khadhraoui et al., 2018). The decreased lightness and higher *b** value of DES-E may be attributed to the color imparted by the protein isolates and intact plant pigments in the flour.

5.4.4.2. Surface hydrophobicity (S_0)

The surface hydrophobicity is related to the number of hydrophobic patches on the protein surface when exposed to a hydrophilic environment and provides useful information about the structure-related characteristics of protein molecules (Hu et al., 2015). Hence, it can influence proteins' stability, conformation, and function. Table 5.4 shows no significant differences in pre-treated FBPI compared to DES-C. It can be expected that EP preserves the protein's original structure in contrast to UP. However, DES-U had slightly lower hydrophobicity than DES-C. The UP treatment can cause the unfolding of proteins, exposing the protein's interior hydrophobic core due to the high energy produced through the strong cavitation effect, increasing S_0 (Lee et al., 2016; Zhou et al., 2016). On the other hand, the prolonged cavitation event may augment the unfolding threshold, which is conducive to reaggregation, protecting the exposed hydrophobic patches. (Gulzar et al., 2024; Malik et al., 2017). This may explain the slight decrease in the hydrophobicity of DES-U when compared to that of DES-C.

5.4.4.3. Thermal stability

Higher thermal stability in food proteins is crucial, as most food processing is performed under heating conditions that can subsequently alter the quality and functionality of the end product. Since the thermal stability of proteins depends on the processing conditions, high temperatures of denaturation are particularly important for the prolonged processing of foods to achieve the desired structure-functional characteristics. The thermodynamic properties, characterized using DSC, for the onset denaturation temperature (T_o), peak denaturation temperature (T_d), and enthalpy changes (ΔH) of the samples are presented in Table 5.4. Compared to DES-C, no significant difference was observed in pre-treated samples for T_o and T_d. The T_d values of all samples ranged from 99.81 ± 1.81 °C to 102.80 ± 2.85 °C, which are comparable to previously reported values for faba bean

isolates (Badjona et al., 2024). Nonetheless, the enthalpy changes (ΔH) of DES-C and DES-E were almost similar ($P>0.05$), but DES-U showed a significantly lower ΔH. This indicated that the DES-U was less thermally resistant than the DES-C and DES-E. Hence, the UP-induced unfolding of the protein signified fewer intramolecular interactions in the aggregated proteins (Gulzar et al., 2024).

Table 5. 4. The effect of pre-treatment assisted DES extraction on the thermal properties, color, and surface hydrophobicity of the FBPIs

Pre-treatment method	Thermal properties			Color			Surface hydrophobicity (S_0)
	T_o (°C)	T_d (°C)	ΔH (J/g)	L*	a*	b*	
DES-C	54.57 ±1.96[a]	99.81±1.81[a]	205.86±2.97[a]	78.73±0.29[a]	1.70±0.00[a]	15.60±0.20[b]	1.94±0.54[a]
DES-U	52.12±0.56[a]	102.80±2.85[a]	152.03±2.71[b]	75.67±1.45[b]	1.70±0.00[a]	10.07±0.15[c]	1.87±0.27[a]
DES-E	54.14±1.04[a]	101.11±1.43[a]	206.17±3.73[a]	69.50±0.20[c]	1.87±0.15[a]	16.30±0.36[a]	2.01±0.37[a]

Fava bean protein isolates extracted from various pre-treatment-assisted DES extraction methods: *i.e.*, Control sample (DES-C), Ultrasound-assisted fava bean protein isolates (DES-U), and Enzyme-assisted fava bean protein isolates (DES-E). Results are presented as mean ± standard deviation (n=3) and were analyzed by analysis of variance (ANOVA) followed by Tukey's test (n=3). Different superscripts within a column indicate significant differences ($P < 0.05$).

Table 5. 5. The effect of pre-treatment assisted DES extraction on the least gel concentration

Protein ingredients	3						5						7						9					
% /pH	5	8	10	12	15	20	5	8	10	12	15	20	5	8	10	12	15	20	5	8	10	12	15	20
DES-C	S	S	S	S	S	S	S	S	S	S	S	S	×	×	×	S	S	F	×	×	×	S	S	S
DES-U	F	F	F	F	F	F	F	F	F	F	F	F	S	S	S	S	S	S	×	×	×	×	S	S
DES-E	F	F	F	F	F	F	F	F	F	F	F	F	×	S	S	F	F	F	×	×	S	S	S	S

The least gel concentration evaluated at pH 3.0-9.0 and protein concentration (5-20% w/v) of fava bean protein isolates extracted from various pre-treatment-assisted DES extraction methods: *i.e.*, Control sample (DES-C), Ultrasound-assisted fava bean protein isolates (DES-U), Enzyme-assisted fava bean protein isolates (DES-E).

5.4.4.4. Particle size distribution

The effect of UP and EP on the particle-size distribution of FBPI is shown in Figure 5.3. It was observed that DES-U exhibited a monomodal distribution similar to that of DES-C, with the majority of particles concentrated around 43.75 ± 0.49 µm, accounting for approximately 50% of the bulk sample [Figure 5.3(a)]. The volume-weighted mean particle diameter [D (4,3)] of DES-C was 28.33 ± 2.03 µm. The D (4,3) is the volume occupied by the particles and is reported to be highly sensitive to agglomeration, disaggregation, or flocculation (Arzeni et al., 2012). As shown in Figure 5.3(b), the application of UP resulted in a significant change in particle size, with a nearly 2-fold increase (53.50 ± 0.57 µm) compared to DES-C. The formation of soluble aggregates might cause an increase in the particle size of DES-U (Tang et al., 2009), which was agreed upon due to the significantly higher solubility (pH 7.0) of DES-U compared to DES-C, as depicted in Figure 5.6(a). In addition, the effect of turbulence and shear forces upon UP may increase the rate of collision and aggregation, consequently forming unstable aggregates, leading to an increase in the particle size (Jiang et al., 2014). Arzeni et al. (2012) reported that aggregates formed mainly due to non-covalent hydrophobic interactions, which were even confirmed in our study. These results suggest that covalent bonds may not have contributed to the formation of aggregates during the UP. On the contrary, DES-E exhibited a bimodal particle size distribution, with a major and minor peak, characterized by particles with a diameter of around 3.68 ± 0.007 µm, accounting for 50% of the bulk sample. Thus, DES-E showed a nearly 2-fold smaller ($P<0.05$) D (4,3) value than the DES-C. The results are consistent with the findings of Zhang et al. (2025), who concluded that spirulina extract had significantly reduced particle size following the Viscozyme L treatment compared to a single carbohydrase.

In the case of the surface-weighted mean particle size [D (3,2)], DES-C had an average particle size of 11.03 ± 0.57 µm. Similar to the D (4,3) distribution, the D (3,2) behavior exhibited the same trend for all protein samples. DES-U had a significantly higher value (21.8 ± 0.14 µm) compared to DES-C, whereas DES-E exhibited the lowest D (3,2) values (2.34 ± 0.007 µm) compared to both DES-C and DES-U ($P < 0.05$). Overall, results suggested that varying particle sizes obtained from the UP and EP might impact the functionality of proteins.

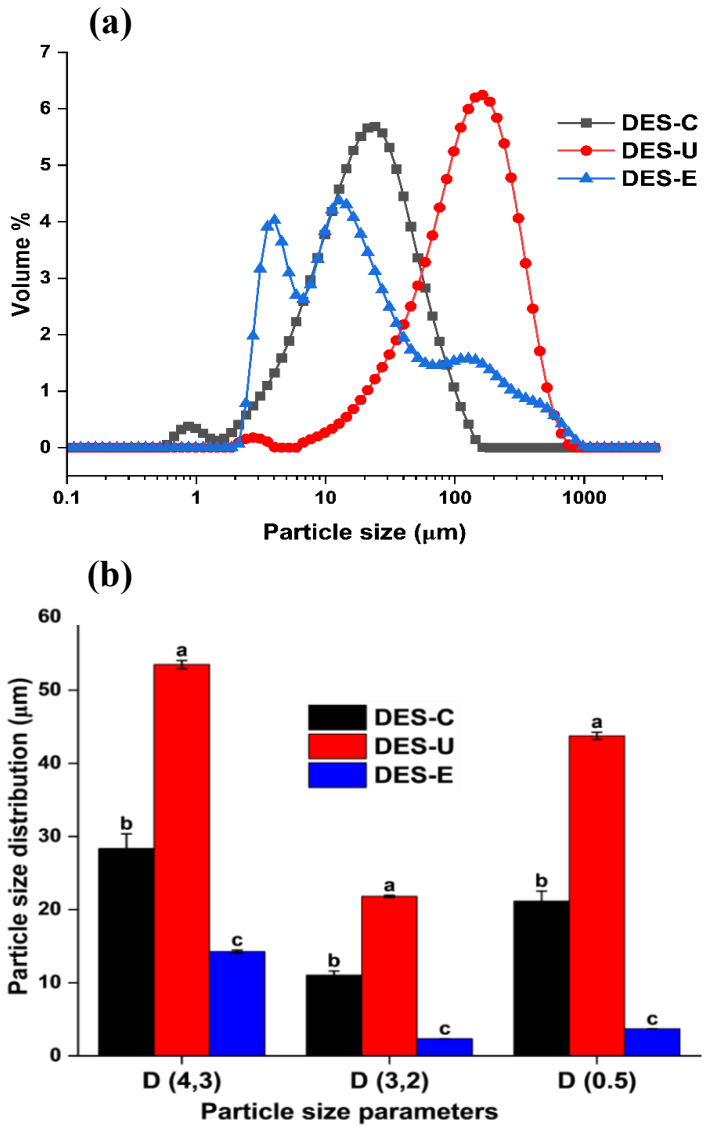

Figure 5. 3 (a) Volume-weighted particle size distribution as a percentage and **(b)** Volume-weighted [D (4,3)], surface-weighted [D (3,2)], and 50th percentile [D (0.5)] mean particle size diameter (μm) of fava bean protein isolates extracted from various pre-treatment assisted DES-extraction, *i.e.,* Control sample (DES-C), Ultrasound-assisted fava bean protein isolates (DES-U), enzyme-assisted fava bean protein isolates (DES-E). All data are presented as means ± standard deviation and were analyzed by analysis of variance (ANOVA) followed by Tukey's test (n=3). Different letters on the error bars indicate the significant difference at $P<0.05$

5.4.4.5. SDS-PAGE

Figure 5.4 illustrates the electrophoresis pattern of proteins isolated from UP and EP-assisted DES extraction of fava beans under non-reducing and reducing conditions. Under non-reducing conditions, the prominent bands were identified in all the samples at molecular weights of ~63, 50, and 37 kDa, corresponding to convicilin, legumin, and vicilin, respectively (Vogelsang-O'Dwyer et al., 2020). Additionally, two minor bands at ~30-35 kDa were visible, possibly corresponding to the minor vicilin subunits (Sharan et al., 2021). Under reducing conditions, the legumin subunit (~50 kDa) dissociated into its characteristic bands, which emerged at approximately 35 kDa and 18–23 kDa, corresponding to the acidic α-legumin subunit and basic β-legumin subunits, respectively (Żmudziński et al., 2021). After the application of UP, under non-reducing conditions, the polypeptide band visible at ~37 kDa (vicilin) was reduced in intensity compared to the DES-C. This demonstrated the partial fragmentation or degradation of vicilin subunits upon sonication. These results aligned with the reported findings of Gulzar et al. (2024). Conversely, EP showed the disintegration of the convicillin identified at ~63 kDa. However, the bandwidth of vicilin in DES-E was broader, and minor vicilin subunits and small molecular weight albumins (~15 kDa) were more visible compared to DES-C and DES-U. This indicated that EP

could lead to the extraction of more vicilin subunits and albumin fractions, suggesting the effective enzymatic hydrolysis of the cell wall by Viscozyme L. In contrast, under reducing conditions, the intensity of both vicilin (~45 kDa) and convicilin (~63 kDa) polypeptide bands of DES-E has been slightly reduced compared to the other counterparts. Additionally, legumin's basic subunits of DES-E had higher band intensity than the other cases. Besides, band intensity across all polypeptides remained unchanged under the reducing conditions. These results revealed that in the current study, UP did not form high-molecular-weight covalently or non-covalently bound aggregates, which typically appear as a smear on top of the electrophoresis gel (Alavi et al., 2021). Overall, slight differences in band intensities were detected between the two pre-treatment methods, which may affect the proteins' functional properties.

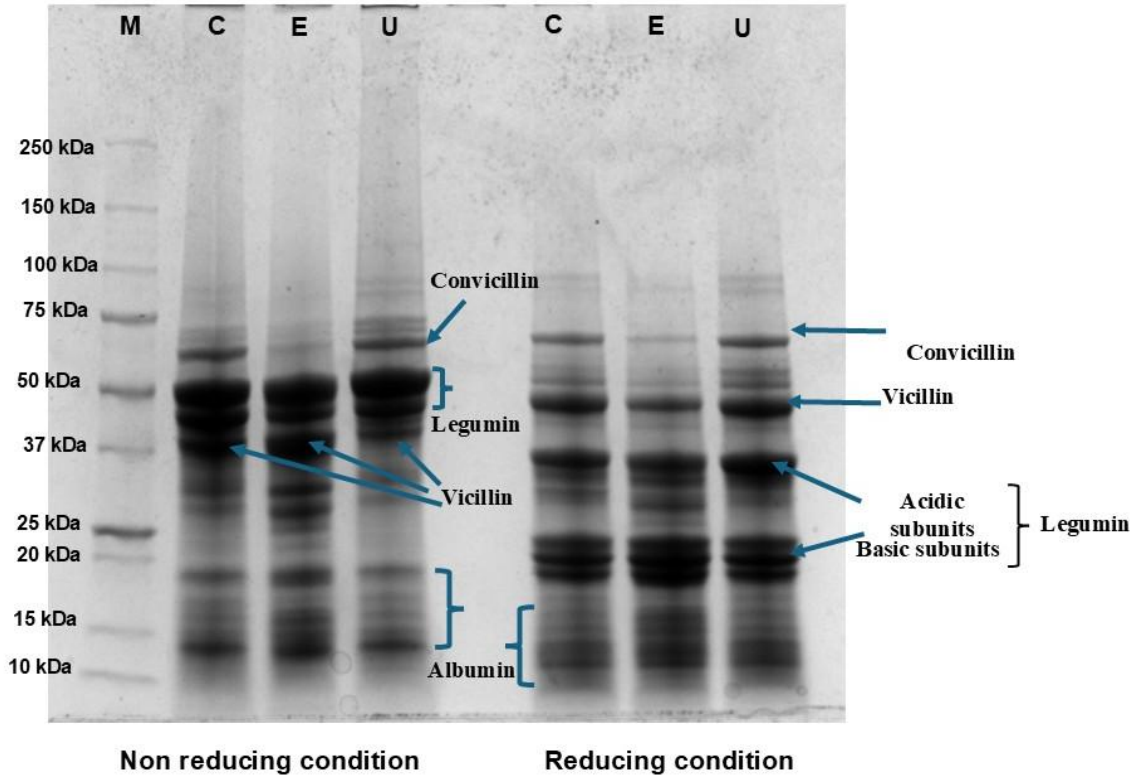

Figure 5. 4. The electrophoresis profile of fava bean protein isolates extracted from various pre-treatment assisted DES-extraction processes, denoted by lane C -control, lane E -Enzymatic pre-treatment, lane U -Ultrasonication pre-treatment, and lane M -molecular weight marker.

5.4.4.6. Changes in secondary structures

The secondary structural components of the FBPIs were characterized by FTIR spectroscopy. **Table 5.5** and **Figure 5.2S** (Appendix 13) illustrate the changes in the secondary structure components of FBPI and their relative percentage, after ultrasonication and enzyme pre-treatments. In the infrared region (400-4000 cm^{-1}) of the FTIR spectrum, the amide I band (1600-1700 cm^{-1}) is used to predict the secondary structural component of proteins. These are primarily due to the C=O stretching vibration (80%), which includes in-plane N-H bending and C-H stretching (Wen et al., 2019). The amide I band represents several overlapping secondary structural components, including α-helix, β-sheets, β-turns, and random coils. The peaks of 1620-1642 cm^{-1} and 1680-1690 cm^{-1}, 1644-1648 cm^{-1}, 1650-1660 cm^{-1}, 1663-1680 cm^{-1}, and 1610-1620 cm^{-1} and 1690-1700 cm^{-1} were assigned to the secondary structure of β- sheets, random coils, α- helix, β-turns and intermolecular β- sheets aggregates (Shevkani et al., 2019; Yang et al., 2015), respectively. The quantification of secondary structural components was performed using a second-derivative peak-fitting procedure, as presented in Table 5.6. Generally, both pre-treatment methods markedly modified the secondary structural components in FBPI compared to the control sample. The UP led to a greater reduction in β-sheet content and a slight decrease in β-turns compared to DES-C. The proportions of other structural components remained relatively unchanged. The reduction of β-sheets suggests that ultrasonication induces the loss of ordered structures due to protein unfolding and disruption of intermolecular hydrogen bonding (Gulzar et al., 2024). The same effects were reported for soy protein isolates, in which β-sheet contents

decreased upon ultrasonic treatment at 200–600 W (Hu et al., 2013). Moreover, the cavitation effects during ultrasonic treatment cause mechanical vibration and strong microstreaming, which break the protein molecules, altering the original secondary structure (Cui et al., 2021). Notably, no studies have been conducted on the changes in DES structure upon ultrasonication. Therefore, when acoustic waves travel through the DES medium, the hydrogen bond network of DES could be disrupted, thereby altering the interaction of proteins with DES components. Thus, protein conformation could be affected. The DES-E also showed drastic changes in secondary structural components. Notably, an appreciable amount of β-sheets and α-helix (~2-fold) and a slight increase of β-turns were observed compared to DES-C. Turker & Isleroglu (2024) demonstrated that Viscozyme L-assisted alkaline extraction resulted in no changes in the secondary structure components compared to the control and Viscozyme L+ ultrasonication-assisted alkaline extraction from cress seeds. It was expected that the minimal changes in the secondary structural components of FBPI obtained from DES-E or similar to DES-C, such as Viscozyme L, would not impact the proteins. However, it can be suggested that when breaking the cell wall through enzymatic hydrolysis, the membrane-bound proteins may undergo physical stress, affecting their structure, consistent with the smaller particle size obtained for the DES-E (Figure 5.3).

Table 5.6. Relative percentage of secondary structural components of FBPI subjected to various pre-treatment methods

Secondary Structure Component	**DES-C**		**DES-U**		**DES-E**	
	Peak center value (cm^{-1})	Relative content (%)	Peak center value (cm^{-1})	Relative content (%)	Peak center value (cm^{-1})	Relative content (%)

β- sheet	1623, 1632, 1680	28.86	1626, 1633, 1681	18.48	1629, 1980	18.92
α-helix	1650, 1660	9.77	1650, 1660	8.76	1650	4.84
β-turns	1667	8.02	1668	5.62	1662	10.54
Intermolecular β-sheets	1692	5.26	1619	5.3	1692	4.67
Random coils	1643	4.38	1643	4.04	1643	3.78

Secondary structure components of fava bean protein isolates extracted from the various pre-treatment assisted DES-extraction, DES-C- control, DES-U- Ultrasonication-assisted DES extraction, and DES-E- Enzyme-assisted DES extraction. All data are presented as means (n=3).

5.4.4.7. Scanning electron microscopy (SEM)

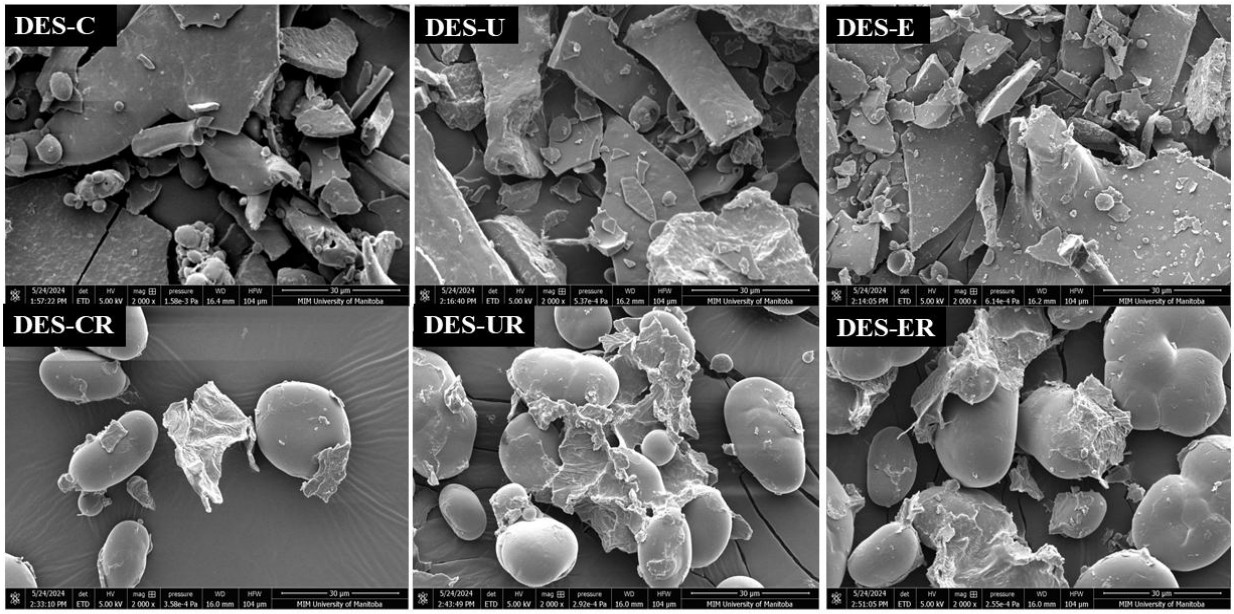

Figure 5. 5. Scanning electron microscopy (×2000) of fava bean protein isolates extracted from the various pre-treatment assisted DES-extraction, DES-C- Control, DES-CR- Corresponding residual after the extraction, DES-U- Ultrasonication-assisted DES extraction, DES-UR- Corresponding residual after the extraction, DES-E- Enzyme-assisted DES extraction, and DES-ER- Corresponding residual after the extraction.

The microstructure of the extracted protein samples and corresponding residual materials after each protein extraction, visualized by SEM, is illustrated in **Figure 5.5**. The visual appearance of the protein extracted by EP was similar to that of DES-C, but DES-U showed slight differences. The surface characteristics of all protein isolates revealed irregular, square-shaped sheet-like fragments resulting from the freeze-drying effect (Dent & Maleky, 2023). The protein surface of DES-C and DES-E was observed to have a smooth appearance, supported by Görgüç et al. (2019), who reported that Viscozyme L-treated protein extraction from sesame bran had a less defective structure than alcalase-treated bran. In contrast, DES-U exhibited a slight wrinkle on the protein

surface and a smaller, aggregated spherical structure, plausibly due to the cavitation effect (Kalla-Bertholdt et al., 2023). The images obtained in our study are similar to those of Byanju et al. (2020) and Jiang et al.(2014). After each protein extraction, the residual materials were collected and visualized in SEM to analyze the structural features of the cell wall and starch granules. There was a deposition of debris, likely cell wall material, on the starch granules, which suggested cell wall breakdown. This was more prominent with the UP (DES-UR), followed by EP (DES-ER) and DES-CR. However, the starch granules from the EP appear much larger and have an oval shape, but apparent cracks or fissures are visible on the surface, unlike those in DES-CR and DES-UR. This may be attributed to the enzymatic hydrolysis of the cell wall, which facilitates the release of starch granules embedded in the protein matrix (Bhattarai et al., 2018).

5.4.5. Effect of pre-treatments on the techno-functional properties of FBPI

5.4.5.1. Protein solubility

Protein solubility is a reliable index for predicting protein functionality, as it is a prerequisite factor for determining the emulsification, foaming, and gelation properties. The solubility of FBPI extracted by UP and EP-assisted DES extraction was assessed in the range of pH 3.0 to pH 9.0. range. Figure 5.6 (a) shows that protein solubility was improved through the pre-treatments across all pH ranges studied when compared to DES-C. The solubility of proteins was higher in both acidic and alkaline conditions, with the latter exhibiting a more pronounced effect. The solubility of DES-C at pH 3.0 was 72.77 ± 2.12%, which increased significantly with the UP (87.64 ± 3.99%). However, no substantial difference ($P>0.05$) was observed between DES-E (70.97 ± 6.97%) and the control. The minimum solubilities of all FBPIs were observed at pH 5.0, corresponding to their isoelectric point, due to a decrease in protein net charge. At pH 7.0 and 9.0, DES-E had a significantly higher solubility, followed by DES-U and DES-C. Many factors

can affect protein solubility, including protein molecular size and structure (Wang et al., 2020). Even though the particle size of the DES-U was higher (Figure 5.3) than that of the other counterparts, the partial folding of protein, accompanied by UP, could have increased the interaction between protein and water molecules by exposing polar amino acid residues, thus increasing protein solubility (Jiang et al., 2014). Additionally, the reduction of β-sheets observed in DES-U (Table 5.5) may have contributed to the increased solubility. These results are in line with the results of previous studies, which showed that ultrasound treatment caused an increase in the protein solubility pea protein (Wang et al., 2020), fava bean (Gulzar et al., 2024), black bean (Jiang et al., 2014), and cress seed protein (Turker & Isleroglu, 2024). Interestingly, the remarkably lower particle size of the DES-E (Figure 5.3) had a profound impact on the increased solubility. Additionally, the reduction in both β-sheet and α-helix content of DES-E may also be attributed to enhanced protein solubility.

5.4.5.2. Water and Oil holding capacity

Proteins' water-holding capacity (WHC) and fat-holding capacity (OHC) are important parameters that improve juiciness, thickening, and viscosity of food products. The amount of oil a food can hold affects flavor retention, such as in meat analogs, soups, and bakery products (Dabbour et al., 2018). The WHC and OHC of FBPI obtained using pre-treatment assisted DES extraction are shown in **Figure 6(b).** With ultrasonication, the WHC (63.56 ± 0.78%) was significantly reduced ($P<0.05$) compared to DES-C (73.64 ± 4.51%), but DES-E showed a substantially higher value (98.84 ± 1.59%). It might be anticipated that with a higher solubility of DES-U than that of the control, it should have a higher WHC. Therefore, the capacity of physical entrapment of water within the DES-U may be related to the microstructural alteration caused by ultrasonication. The weaker intramolecular interaction, such as hydrophobic interactions between

the aggregates, may play a key role in the lower water entrapment within the protein structure, thus revealing fewer binding sites for water (Kaur & Singh, 2007). On top of that, the larger particle size of the proteins may result in lower water binding within the protein matrix as the surface area increases. The significantly higher WHC of DES-E can be attributed to its higher solubility, resulting from the more polar amino acid residues on its surface and its smaller particle size. These results are consistent with previously reported data (Resendiz-Vazquez et al., 2017). Contrary to the current study, it was shown that the WHC of fava bean protein was increased after ultrasonication (Narale et al., 2024).

The OHC, however, showed no differences between the DES-C and the DES-U, suggesting a similar distribution of non-polar amino acids, regardless of their particle size differences. For the DES-E, a notably higher OHC ($P<0.05$) than those of DES-C and DES-U may be explained by the presence of more non-polar side chains that bind the hydrocarbon chains of fats (Sathe et al., 1982) and is supported by the lower particle size observed in this study.

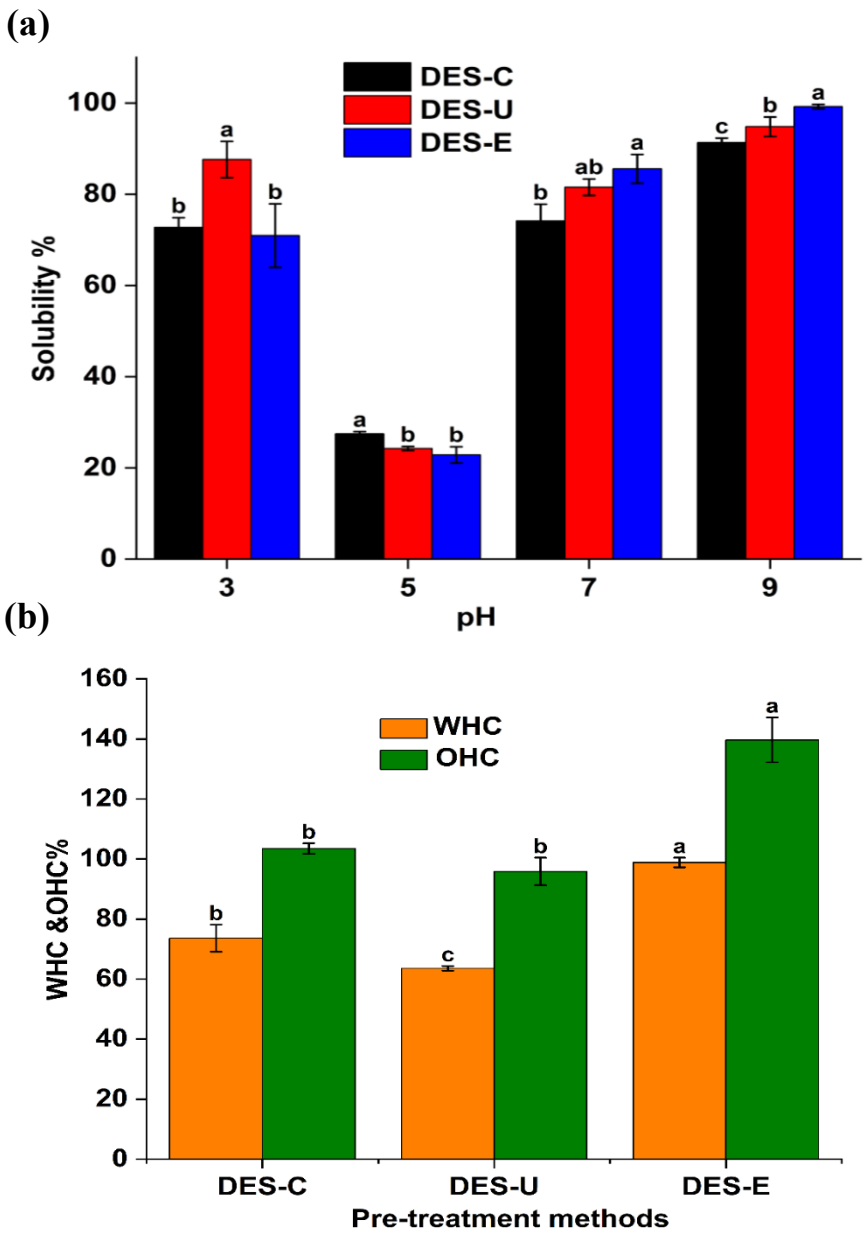

Figure 5. 6(a)- Solubility percentage as a function of pH, and **(b)**- Water holding capacity (WHC) and oil holding capacity (OHC) of fava bean protein isolates extracted from various pre-treatment-assisted DES-extraction processes, *i.e.,* DES-C- Control, DES-U- Ultrasonication-assisted DES extraction, and DES-E- Enzyme-assisted DES extraction. All data are presented as means ±

standard deviation and were analyzed by analysis of variance (ANOVA) followed by Tukey's test (n=3). Different letters on the error bars indicate the significant difference at $P<0.05$.

5.4.5.3. Emulsification properties

The emulsification properties of plant proteins are crucial for their applicability in a wide range of food products, including butter, margarine, mayonnaise, and meat alternatives. The amphiphilic nature of the proteins allows them to form a cohesive, viscoelastic film at the water-oil interface, surrounding oil droplets and acting as an emulsifier to stabilize various food systems. The emulsifying activity index (EAI) and the emulsion stability index (ESI) of the FBPIs subjected to varying pre-treatments are presented in Figure 5.7(a). The EAI of DES-C, DES-U, and DES-E was 20.45 m^2/g, 19.68 m^2/g, and 22.51 m^2/g, respectively, and was not significantly different from each other ($P > 0.05$). This indicated that neither pre-treatment improved the emulsion activity index compared to the control sample. Generally, the presence of more hydrophobic groups on the surface leads to a considerable decrease in the oil/water barrier surface tension, thereby facilitating fast structural reorientation and adsorption of protein molecules at the interface without emulsion droplet break up (Wang et al., 2017). Despite the higher solubility of pre-treated FBPIs, the proteins' surface hydrophobicity will likely play a crucial role in enhancing their emulsification properties. Since no significant difference in surface hydrophobicity was observed for all the FBPIs, this may be a plausible reason why there were no improvements in the EAI for DES-U and DES-E when compared to DES-C. As investigated by researchers, it was found that sonication of millet protein at 18.4 W/cm^2 for 5 to 12.5 min did not alter its emulsifying properties; however, 20 min led to a significant improvement (Nazari et al., 2018).

The emulsification stability index (ESI), however, varied significantly ($P < 0.05$) among the samples; the DES-E had a higher ESI than DES-U and DES-C. Besides the significant

difference in ESI among samples, the value varied from 11.21 min to 11.75 min, showing almost the same values. This means neither UP nor EP can modify the protein structure to form a strong viscoelastic film at the oil-water interface through balanced hydrophilic and hydrophobic intermolecular interactions under the experimental conditions employed in this study. Moreover, the fragmentation of vicilin and convicilin protein subunits, as evident in the electrophoresis profile (Figure 5.4) and the reduction in β-sheet content (Table 5.5), may be responsible for the weaker stabilization effect of the proteins at the oil-water interface.

5.4.5.4. Foaming properties

Foaming properties involve the diffusion of protein molecules through the bulk phase, reorganization, and stabilization at the air-water interface. Similar to emulsifying properties, foaming properties are influenced by size, structural flexibility, surface hydrophobicity, structural orientation, and the degree of denaturation of protein molecules (Rahman & Lamsal, 2021). The foaming capacity (FC) and foaming stability of FBPIs, as affected by varying pre-treatment-assisted DES extraction, are presented in Figure 5.7(b). Compared to their emulsifying properties, all FBPIs exhibited similar behavior, showing no significant changes ($P > 0.05$) in FC among the samples, which ranged from 51.50% to 53.83%. This also suggested that the hydrophobicity of proteins was a key indicator for establishing good foaming properties. However, for both DES-U (~50 min) and DES-E (~66 min), it was demonstrated that the FS was significantly lower ($P < 0.05$) than that of DES-C (~93 min). Moreover, considering DES-U and DES-E, the former had a lower FS ($P<0.05$) than the latter. As described earlier in the context of emulsifying properties, molecular weight, and flexibility levels below the optimal range can affect the strong foaming stability at the air-water interface (Gulzar et al., 2024). Furthermore, the UP may have resulted in

a lower FS due to the larger size of the aggregates (higher particle size, Figure 5.3) and altered conformational changes, specifically a reduction in β sheets (Table 5.5).

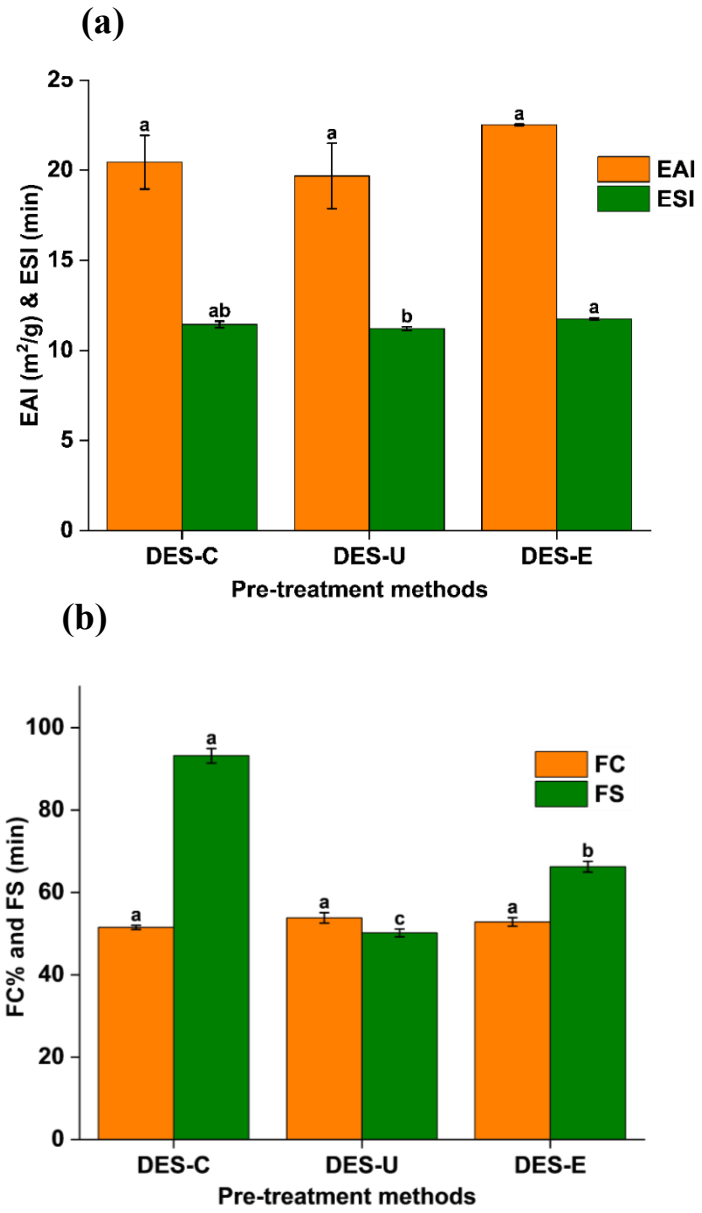

Figure 5. 7 (a)- Emulsification activity index (EAI) at pH 7.0 and protein concentration of 1% w/v, and **(b)-** Foaming capacity (FC) and foaming stability (FS) under the same conditions, of fava bean protein isolates extracted from various pre-treatment-assisted DES-extraction. DES-C- Control, DES-U- Ultrasonication-assisted DES extraction, and DES-E- Enzyme-assisted DES

extraction. All data are presented as means ± standard deviation and were analyzed by analysis of variance (ANOVA) followed by Tukey's test (n=3). Different letters on the error bars indicate significant differences at $P<0.05$.

5.4.5.5. Least gel concentration (LGC)

Gelation is one of the most important functional characteristics of proteins, with diverse applications in foods such as yogurts, cheeses, desserts, eggs, and meat products to achieve desirable textural and sensory properties. The protein's molecular structure, including the unfolding of α-helix and β-sheets, primarily influences its gelation properties (Bangar et al., 2022). Generally, gelation is characterized by the denaturation, aggregation, and formation of a three-dimensional network by aggregated protein molecules at a critical protein concentration and pH (Tang et al., 2024). The LGC of FBPIs, evaluated at pH levels ranging from 3.0 to 9.0 with protein concentrations of 5-20% w/v after varying pre-treatment assisted DES extractions, is tabulated in Table 5.5. The LGC indicates the gelation capacity; the lower the LGC, the better the gelling ability of proteins. Both the pre-treatments induced the improved gelling ability of FBPI at different pH levels studied. DES-C showed the LGC at 5% w/v protein concentration (PC) at pH 3.0 and 5.0 (as a soft gel based on our categorization), whereas, at pH 7.0 and 9.0, it increased to 12% w/v PC. The lower electrostatic repulsion between the protein aggregates at lower pH and PC was probably conducive to gel formation. However, as previously reported in our study (Chapter 4), the firm gel was only produced at a pH of 7.0 with 20% w/v PC. In contrast, DES-U and DES-E produced firm gels across all the PC investigated at pH 3.0 and 5.0. The aggregated proteins from acoustic cavitation generated by ultrasonication may have enhanced hydrophobic interactions during heating, further reinforcing the gel via covalent bonds (Hu et al., 2013). In particular, the particle size of the DES-E may exert a significant influence on gel network formation, as supported by its

high protein content (>95%), solubility [Figure 5.6(a)], and water-holding capacity [Figure 5.6(b)] within the microstructure of the gel network. At pH 7.0, however, DES-U did not produce a firm gel across all the PC, but soft gels. This can be explained by the loss of electrostatic balance between protein aggregates, resulting from the combined effect of the higher particle size, which leads to weaker intermolecular interactions (Zhao et al., 2022). Nonetheless, DES-E exhibited self-standing gels with firmer characteristics even at pH 7.0 in the PC 12-20% w/v range. Notably, the higher solubility and greater particle size reduction of DES-E can lead to a tighter and more uniform gel structure formation (Zhao et al., 2022). At pH 9.0, the protein is highly soluble; therefore, none of the FBPIs produce firm gels at the given PC. However, soft gels were formed at 12%, 15%, and 10% w/v for DES-C, DES-U, and DES-E, respectively, as indicated by the LGC.

5.5. Conclusion

This study investigated the protein yield and structural-functional properties of fava bean protein isolates produced using enzymatic and ultrasonication pre-treatment-assisted DES extraction. Both pre-treatments significantly increased the protein yield by approximately 27% with UP and 8% with EP. The high yield increase by ultrasonication could be applied to the industrial scale-up as a modified, sustainable DES extraction method. DES-U's more than 95% protein content potentially enables the upgrading of underutilized fava beans to produce high-quality plant protein ingredients with nearly zero fat content, supporting value-added functional food formulations. FBPIs from non-thermal processes, such as UP and EP, can be used to modify the structure-functional attributes, including solubility, water and oil holding capacity, and gelation, thereby offering clean-label food products. Notably, the improved gelling capacity over a wider pH range would expand the range of various plant-based novel food processing options. However, the improvement of emulsification and foaming properties was not observed with the

application of pre-treatments in this study, possibly due to the modification of the protein structure by reducing the secondary structural components, such as β-sheets and α-helix. Thus, further studies are warranted in addition to the valuable insights provided in this study. Moreover, assessing the feasibility and cost-effectiveness of these non-thermal methods is necessary for implementing them on an industrial scale.

Connection to Chapter 6

In the plant system, proteins are bound to various compounds, including polysaccharides, fiber, starch, and polyphenolic compounds. Additionally, proteins are present in various forms within the cell, where structural proteins are located in the cell wall, while storage proteins are found in the cell matrix. Thus, a rigid and dense cell wall impedes protein extraction efficiency. The ultrasonication and enzymatic pre-treatment-assisted DES protein extraction successfully resolved these barriers, enhancing the liberation of proteins into the extraction medium. Both ultrasonication and enzymatic pre-treatment with optimization improved the protein extraction yield by ~27% and 8%, respectively, compared to the control sample. Protein purity, however, did not change significantly in the ultrasound-treated fava bean protein (~88%) compared to the control (~90%), whereas enzymatic treatment significantly enhanced protein purity (>95%). The solubility, water-holding capacity, oil-holding capacity, and gelling capacity were significantly improved with the pre-treatments, indicating potential for scale-up at the industrial level for novel food applications. Despite the promising advantages revealed in this research study, the viscosity of DES may still hinder its practical applications in industrial processes. The high viscosity of DES hinders mass transfer rates, thereby impacting extraction efficiency. Moreover, high viscosity limits the flow properties of the solvent, impacting the pumping system in the industrial process, which subsequently requires high energy for protein extraction. Thus, adding water can considerably reduce viscosity and improve the extraction efficiency of proteins by increasing the mass transfer rate. Nevertheless, adding water can drastically alter the physiochemical properties of DES, which may impact the protein and DES interactions. Therefore, it is worthwhile in Chapter 6 to investigate the effect of water addition on the physical and nanostructural changes of the DES and the concurrent impact on protein yield, protein content, and the techno-functional properties

rendered by different water percentages in the DES system. This provides novel insights into understanding how the DES system can be tuned by adding water, with its changing physicochemical properties, for the development of specific functional properties of fava bean protein suitable for specific food applications.

Chapter 6

Effect of water addition on structure and functionality of fava bean proteins extracted by choline chloride and glycerol-based deep eutectic solvent system

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6.1. Abstract

In the present study, the structure and functionality of fava bean proteins extracted by a hydrated choline chloride (ChCl) and glycerol (Gly)-based deep eutectic solvent (DES) system were evaluated. The impact of water addition on DES's physicochemical properties and nanostructure was analyzed using FTIR and NMR spectroscopy. The dilution of DES, particularly at a 10 wt% water concentration, significantly reduced viscosity by 85% and increased conductivity fourfold ($P < 0.05$). The results demonstrated that water has strong interactions with DES components, and dilution with water by 10 wt% caused minimal disruption to the DES nanostructure. However, subsequent water addition gradually weakened the hydrogen bond network up to 40 wt% while still preserving the supramolecular structure to some extent. Further addition of water (>40 wt%) resulted in the complete dissociation of DES components. These physicochemical and structural changes led to significant differences in protein yield (PY), protein content (PC), and the structure-functional properties of the extracted proteins. PY significantly increased as the water content in DES rose from 0% (29.15 ± 3.13%) to 100 wt% (83.83 ± 1.08%). Proteins extracted with 10 wt% water showed a higher percentage of β-sheets and a lower α-helix content. PY increased significantly as the water content in DES increased from 0% (29.15 ± 3.13%) to 100 wt% (83.83 ± 1.08%). Proteins extracted with 10 wt% water had a higher β-sheet content and a lower α-helix content. Moreover, distinct polypeptide bands (SDS-PAGE) differences were observed in proteins extracted with water contents of 10, 20–50, and >50 wt%. These variations in polypeptide bands may be linked to differences in the techno-functional properties of the extracted proteins.

6.2. Introduction

Deep eutectic solvents (DESs) are a novel class of green solvents introduced by Abbott et al. (2003). DESs are considered potential alternatives for conventional solvents and ionic liquids (ILs) owing to their sustainable characteristics, low toxicity, recyclability, biodegradability, and low cost (Hansen et al., 2021; Prabhune & Dey, 2023). Generally, DESs are synthesized by mixing at least two components at a particular molar ratio, usually a hydrogen bond donor (HBD) and hydrogen bond acceptor (HBA), which are capable of self-associating via an extensive network of hydrogen bonds (HBs), to form a eutectic phase, which is characterized by a larger depression in the melting point than that of individual components (El Achkar et al., 2021). DESs have been commonly described by the general formula $Cat^{+}X^{-}zY$; Cat^{+} is the cation of any ammonium, sulfonium, or phosphonium salt, and X^{-} is the Lewis base, generally a halide anion of the salt; Y represents either a Lewis or Bronsted base, and z is the number of Y molecules (Tomé et al., 2018). The primary interaction between X^{-} and Y occurs through self-associated intermolecular hydrogen bonds (HBs), which are fundamental to DES properties (Smith et al., 2014). However, studies suggest that electrostatic and van der Waals forces also play a role during mixing entropy (Hansen et al., 2021).

The extent of the HB network is associated with DES's macroscopic properties, particularly density and viscosity (Ferreira et al., 2021). Depending on the HBD used, a more extensive HB network could create a strong, cohesive force in the liquid, resulting in a significantly high viscosity solvent (Paiva et al., 2014; Stefanovic et al., 2017). Despite their successful application in various sustainable processes (e.g., biomolecule extraction, enzyme catalysis, and biomass processing) (Liu et al., 2018; Hansen et al., 2021; Smith et al., 2014), DESs face challenges due to

high cost and viscosity. These limitations hinder industrial adoption by restricting fluid flow, solvent transfer, and mass transfer efficiency in extractions.

Water substitution has proven an efficient way to modulate the DES physiochemical properties to tailor the specific industrial application. Adding water can drastically alter the viscosity, density, conductivity, and surface tension, as evidenced by a large amount of reported data (Abranches et al., 2021; Chunyan Ma et al., 2018; Dai et al., 2015). Moreover, in recent reviews, theoretical and experimental investigations have well-explained the changes in DES's nanostructure upon adding water (Elbourne et al., 2021; Hammond et al., 2017; Sapir & Harries, 2020). Interestingly, the amount of water added can be controlled by gradual changes in the DES structure, which can then be tailored to form specific physiochemical properties of DES. This would largely benefit plant protein extraction, where desired techno-functional properties can be adjusted for novel food formulations. The solvation effect of DES with water significantly reduces viscosity, enhancing mass transfer rates and increasing protein yield. Additionally, the use of chemicals to synthesize DES would be substantially reduced, benefiting industrial applications at a lower cost. Few studies have explored the extraction of plant proteins using various DES systems. Examples include oats (Yue et al., 2021), soy (Chen et al., 2021), seabuckthorn seed meal (Lin et al., 2022), pomegranate peel (Hernández-Corroto et al., 2020), bamboo shoots (Lin et al., 2020), canola (Karimi et al., 2024), tiger nut meal (Jiang et al., 2024), evening primrose, and rapeseed meals (Grudniewska et al., 2018), but these studies primarily focused on improving protein extraction, and functionality characterizations (Jiang et al., 2024; Karimi et al., 2024; Yue et al., 2021). Based on our previous studies, it has been demonstrated that DES containing ChCl/Gly at a molar ratio of 1:2 and a water content of 40 wt% (60 wt% DES) resulted in protein content of greater than 90% from fava beans, similar to conventional extraction methods (Hewage et al.,

2024). Moreover, in another study, we demonstrated significantly improved techno-functional properties and a protein digestibility-corrected amino acid score (PDCAAS) from DES-extracted fava bean (Chapter 4). However, to our knowledge, no studies have investigated the effect of water addition and changing the physicochemical properties of DES solvents on the structure-functional properties of fava bean proteins.

Although researchers initially believed that the DES-water mixture formed an aqueous solution, its interactions with water and other macromolecules appear far more complex (Sanchez-Fernandez & Jackson, 2020). Water disrupts the hydrogen bonding network between DES components (Vilková et al., 2020), meaning that water content variations can significantly alter DES's structural and physicochemical properties. Consequently, these changes affect protein-DES interactions, leading to conformational modifications. It is crucial to understand how water addition levels influence DES's physicochemical properties and their impact on protein structure-function relationships. This knowledge enables the industry to tailor DES properties to produce specific functional proteins suited to targeted food applications. This study hypothesizes that adding water to the DES would change the protein extraction yield, purity, and techno-functional properties. Therefore, this work aimed to evaluate the effect of water addition in ChCl/Gly DES on the structure-functional properties of fava bean proteins.

6.3. Materials and methodology

6.3.1. Materials

Snowbirds dehulled dried fava beans (non-tannin variety) were purchased from Prairie Fava, MB, Canada. SDS-PAGE electrophoresis chemicals were purchased from Bio-Rad Laboratories Ltd. (Mississauga, ON, Canada). Benzene D_6 was purchased from MilliporeSigma

Canada Ltd. (Oakville, ON, Canada). Unless otherwise stated, all other chemicals were sourced from Fisher Scientific (Ottawa, ON, Canada).

6.3.2. Methodology

6.3.2.1. Synthesis of DES

DES was synthesized based on the method adopted in our previous study (Hewage et al., 2024). Briefly, ChCl and Gly were mixed in a beaker with a molar ratio of 1:2. The resulting mixture was heated at 80 °C (200 rpm) until a clear, transparent liquid was formed. Then, it was allowed to cool to room temperature (22 °C), and the prepared DES was substituted with an appropriate amount of Milli-Q water, ranging from 0 to 100 wt%, as indicated in Table 6.1.

Table 6. 1. Substitution of water with ChCl/Gly DES

Treatment	Water substitution wt%	DES wt %
1	0	100
2	10	90
3	20	80
4	30	70
5	40	60
6	50	50
7	60	40
8	80	20
9	100	0

ChCl- choline chloride, Gly- Glycerol

6.3.2.2. Physiochemical Characterization of DES

The shear viscosity of DES with varying water contents was measured using a TA Discovery HR-20 rheometer (TA Instruments, DE, USA) equipped with a parallel plate geometry (40 mm) and a 1 mm gap, at a constant shear rate of 1 s^{-1} and 25 °C. Density was measured (g/mL) by calculating the mass (g) and volume (mL) at an ambient temperature of 22 °C. The conductivity was measured using a conductivity meter (Model STAR A2120, Thermo Scientific, Beverly, MA, USA) at 22 °C. pH was recorded using a pH meter (Accumet AE150, Fisher Scientific, Ottawa, ON, Canada) at 22 °C. Fourier-transformed infrared spectroscopy (FTIR) was recorded on an INVENIO S (Bruker Scientific LLC, MA, USA) ATR-FTIR at room temperature (22 °C) with a resolution of 4 cm^{-1} using 120 scans, ranging between 400-4000 cm^{-1}. The nuclear magnetic resonance (NMR) experiments were conducted on a Bruker Ascend 600 spectrometer (Bruker BioSpin AG, Fällanden, Switzerland), operating at 600.27 MHz for proton nuclei and 150.938 MHz for carbon nuclei. Each sample was run with a probe temperature of 298.0 K within 24 hrs of preparation. Two different scans, proton nuclear magnetic resonance (^{1}H-NMR) and nuclear overhauser effect spectroscopy (NOESY), were performed for structure determination. The proton was run with a 65.5 kHz time domain, a 90 ° pulse width of 10 µs, a spectral width of 16 ppm, and a relaxation delay of 5 sec. The number of scans produced was 8, with 2 dummy scans having an acquisition time of 1.5 min. The spectra were processed using MestReNova version 12.0.0-20080.

6.3.2.3. Protein extraction

The protein extraction was done using our previously optimized conditions for fava beans with slight modifications (Hewage et al., 2024). Before the extraction, dried fava beans were milled using a Prater-Sterling Impact Mill (M-21, Prater Industries, Bolingbrook, IL, USA) with a 0.2 mm screen. The fava bean flour (FBF) was mixed with DES at predetermined water levels, as

shown in Table 6.1, followed by homogenization at 20,000 rpm for 2 min (Model 850, Fisher Scientific, Ottawa, ON, Canada). Protein was extracted using a circulating water bath (Model 6200 H7, Fisher Scientific Inc., Pittsburgh, PA, USA), and the temperature was maintained at 50 °C for 1 hrs (200 rpm). Then, the extractant was centrifuged (17,709 g, 10 min, 4 °C), dialyzed (3.5 kDa) at 4°C for 5 days, freeze-dried, and stored at -20 °C for further analysis. Protein yield (PY) and protein recovery rate (PR) were calculated using the following equations.

$$PY\%\ (DW) = Ep\ \times P/Rp\ \times 100 \quad (1)$$

$$PR\ \%\ (DW) = \ (Ep\ \times P)/Rw\ \times 100 \quad (2)$$

Where *Ep* is the Weight (g) of the extracted protein, *P* is the percentage of purity of the extracted protein, *Rp* is the Protein content (g) in the initial raw material, and Rw is the weight (g) of the initial raw material. All calculations were on a dry weight (DW) basis.

6.3.2.4. Proximate composition of extracted proteins

Moisture, Crude protein, Crude fat, and ash content were determined according to the AOAC methods (AOAC, 2000). The crude protein content was calculated using the conversion factor of 6.25 (Karaca et al., 2011). The total carbohydrate (CHO) contents were calculated using the difference method.

6.3.2.5. Physiochemical characterization of extracted proteins

6.3.2.5.1. Surface hydrophobicity (H_0)

The surface hydrophobicity (H_0) was measured using 1-anilino-8-naphthalene sulfonate (ANS) probe according to the method outlined by Karaca et al. (2011). The protein stock solution,

prepared in 0.1 M phosphate buffer at pH 7.0, was used to create a series of diluted protein concentrations (50-250 μg/mL) with the same buffer. 250 μL of protein solution was mixed with 20 μL of ANS solution (0.8 M) in a microplate, and the fluorescence intensity (FI) was measured using a spectrofluorometer (Jasco-FP-6300, Tokyo, Japan) at excitation and emission wavelengths of 390 nm and 470 nm, respectively. The H_0 was calculated using the slope of the FI versus protein concentration.

6.3.2.5.2. Particle size distribution

The particle size distribution of protein dispersions was performed using static laser light diffraction (Mastersizer 3000, Malvern Instruments Ltd, Malvern, UK), following the method described by Liu et al. (2021) with alterations. Before analysis. Protein ingredients were then dispersed in a phosphate buffer (pH 7.0) at a concentration of 1% (w/v) and shaken overnight at 4 °C. After samples were equilibrated at room temperature (22 °C), they were introduced into the Mastersizer equipped with a liquid dispersing unit. The laser obscuration of ~12% was used, along with the particle refractive index, absorption index, and dispersant refractive index of 1.45, 0.1, and 1.33, respectively, to achieve the volume-weighted mean particle diameter D (4,3) and surface-area weighted mean particle diameter D (3,2) of protein samples.

6.3.2.5.3. Attenuated total reflectance Fourier-transformed infrared spectroscopy (ATR-FTIR)

Infrared spectra of protein fractions were recorded using an FTIR spectrometer (INVENIO S, Bruker Scientific LLC, MA, USA) equipped with an Attenuated total reflectance (ATR). The 4000-400 cm^{-1} spectra were recorded (120 scans at 4 cm^{-1} resolution) and subjected to second derivative (SD) analysis after the normalization of date and the baseline reduction of amide-I (1700–1600 cm^{-1}) region, using the Savitzky-Golay smoothing function from the Origin 2024 software (OriginLab Corporation, MA, USA). The multiple peak-fitting procedure was performed

under the Gaussian model to identify the secondary structure components using the same software mentioned above.

6.3.2.5.4. SDS-PAGE

The SDS-PAGE was performed according to the method outlined by Laemmli (1970) with modifications described by Aluko & McIntosh (2004). A stock solution of fava bean protein was prepared at a concentration of 6 mg/mL. 1 mL of protein solution, with a final concentration of 4 mg/mL, was mixed in equal volumes with Laemmli buffer, either with or without β-mercaptoethanol. Thereafter, the sample was boiled at 90 °C for 5 min. Separate reduced and non-reduced electrophoresis was performed using a 4-15% Mini-Protean TGX precast gel in a Mini-Protean II electrophoresis unit (Bio-Rad Laboratories Inc., CA, USA) at a constant voltage of 150 V. The gel was stained for 2 hrs with Coomassie Brilliant Blue R-250, then destained for 2 hrs. The stained protein bands were imaged using the Biorad ChemiDocTm Imaging system (Bio-Rad Laboratories Inc., CA, USA).

6.3.2.5.5. Differential scanning spectroscopy (DSC)

The thermal properties of the protein samples were determined using a DSC (Model DSC2500, TA Instruments-Waters LLC, DE, USA) following the modified method of Lan et al. (2020). In brief, sample moisture was removed using a P_2O_5-containing hermetically sealed desiccator for 7 days. Approximately 3-5 mg of protein was loaded into T-Zero hematic aluminum pans and hermetically sealed. The thermal behavior of the samples was recorded at a heating rate of 10°C/min from 30 to 250°C, with the sealed empty pan serving as a reference. The generated thermogram was analyzed to determine the onset temperature (T_o), Peak denaturation temperature (T_d), and enthalpy of denaturation (ΔH) using TA Universal Analysis 2000 software (Model DSC2500, TA Instruments-Waters LLC, DE, USA).

6.3.2.6. Functional properties of extracted protein fractions

6.3.2.6.1. Protein solubility (PS)

PS was determined according to the method described by Krause et al. (2023) with modifications. The dissolved protein isolates in Milli-Q water (0.1 g/20 mL) were hydrated overnight at 4 °C, and pH was adjusted to different pH levels (3.0 to 9.0) using 1 M NaOH or 1 M HCl. Then, the protein solution was stirred at 22 °C and 200 rpm for 1 hrs, followed by centrifugation at 15,000 g for 15 min at 4 °C. The soluble protein content in the supernatant was determined using the Kjeldahl method (AOAC, 2000). The solubility percentage was calculated using equation (3).

$$Solubility\ \% = \frac{Soluble\ protein\ in\ supernetant\ (g)}{Total\ protein\ in\ inital\ sample\ (g)} \times 100 \quad (3)$$

6.3.2.6.2. Foaming capacity (FC) and foaming stability (FS)

FC and FS were determined using a modified method by Adebiyi and Aluko (2011). Briefly, an aliquot of a 1% protein solution (0.2 g/20 mL) was prepared in a measuring cylinder using 0.1 M phosphate buffer at pH 7.0. The foam was generated using a homogenizer (11,000 rpm for 2 min) (Model 850, Fisher Scientific, Ottawa, ON, Canada) equipped with a 20 mm probe. Foam volume was recorded after 10 sec as the initial foam volume (V_0) and 30 min after standing at room temperature (22 °C) (V_1). FC and FS were calculated using equations (4) and (5).

$$FC\% = \left(\frac{V_0}{20mL}\right) \times 100 \quad (4)$$

$$FS\% = \left(\frac{V_1}{V_0}\right) \times 100 \quad (5)$$

6.3.2.6.3. Emulsification activity index (EAI) and emulsification stability index (ESI)

Both EAI and ESI were determined according to Hu et al. (2019) with some modifications. 10 mL of protein solution (1% w/v in 0.1 M phosphate buffer, pH 7.0) was mixed with 5 mL of canola oil. The mixture was mechanically homogenized at 20,000 rpm for 1 min (Model 850, Fisher Scientific, Ottawa, ON, Canada). 50 mL of the pre-emulsion was diluted in 5 mL of 0.1% (w/v) SDS solution, and the absorbance was measured at 500 nm using a UV-Spectrophotometer (Genesys 150, Madison, USA) at times 0 (A_0) and 10 min (A_{10}). The EAI and EAS were calculated using equations (6) and (7).

$$EAI\ (m^2/g) = \frac{2 \times 2.303 \times A_0 \times N}{c \times \varphi \times 10000} \quad (6)$$

$$ESI\ (\text{min}) = \frac{A_0}{\Delta A} \times t \quad (7)$$

Where A_0 is the absorbance of the diluted emulsion immediately after homogenization, N is the dilution factor (×100), c is the weight of protein per volume (g/mL), φ is the oil volume fraction of the emulsion, ΔA is the change in absorbance between 0 and 10 min (A_0–A_{10}), and t is the time interval, 10 min.

6.3.3. Statistical Analysis

All analyses were carried out in triplicate, and the data reported were the means of triplicate observations with standard deviations (SD), except for DSC and Viscosity analysis, which were performed in duplicate. The data were subjected to a one-way analysis of variance (ANOVA), followed by the Tukey test ($P < 0.05$) using MINITAB statistical software (MINITAB version 20, LLC, PA, USA).

6.4. Results and discussion

6.4.1. Effect of water addition on DES physiochemical properties

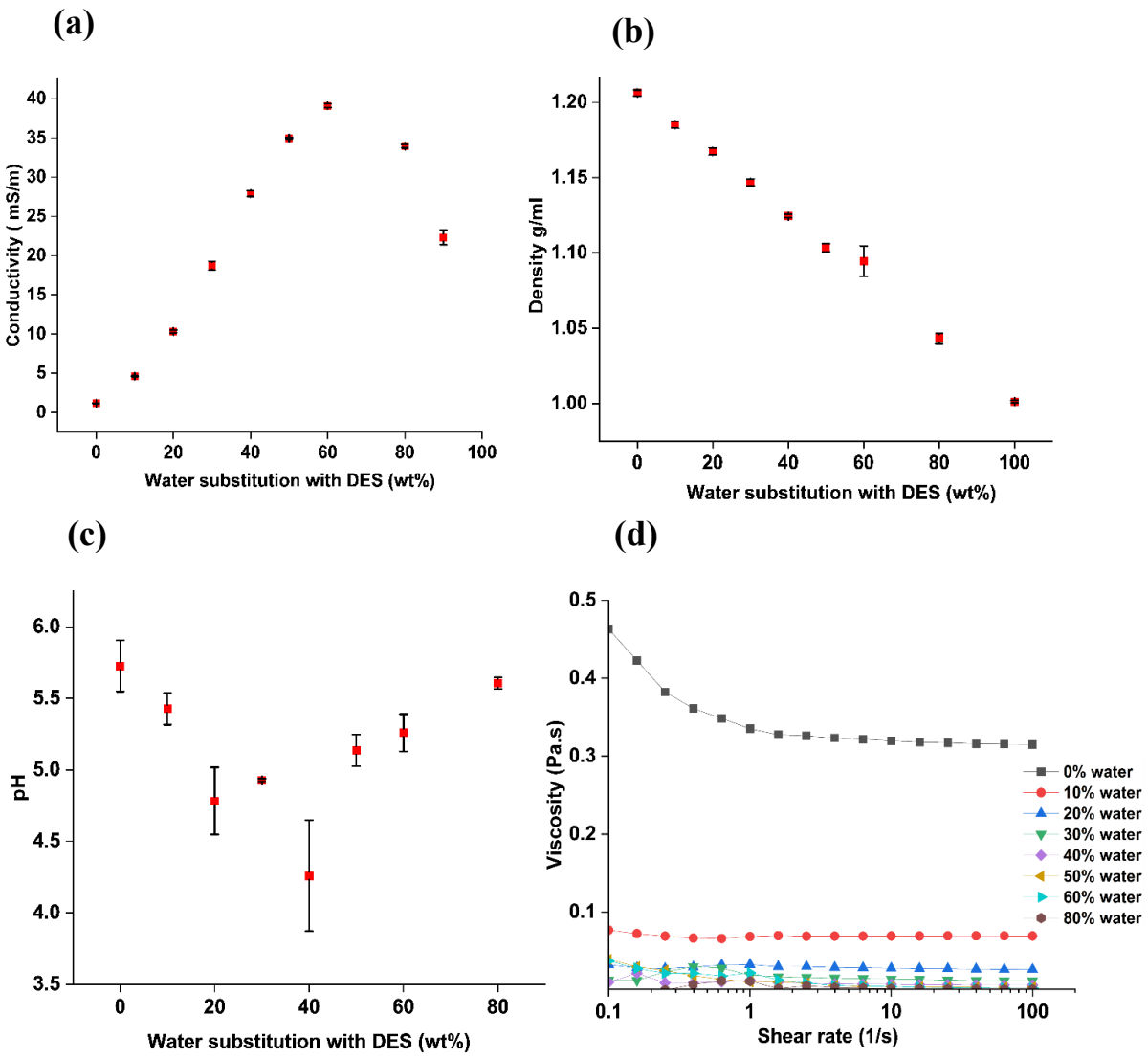


Figure 6. 1 (a) Ionic conductivity, (b) Density, (c) pH, and (d) shear viscosity of the ChCl:Gly (1:2) DES system with varying water content (90-100 wt%). All data were analyzed by nalysis of variance (ANOVA) followed by Tukey's test (n=3), and the results are presented as mean ± standard deviation.

Table 6.1S (Appendix 14) represents the four main physical properties: density, viscosity, pH, and conductivity of the DES solutions prepared. All physical properties varied significantly ($P < 0.05$) among the DES with different water contents. Although density and pH were significantly different within DES's first 10 wt% water content (0-10% w/w), the variation was not much larger. In contrast, ionic conductivity (IC) increased by a factor of 4, while viscosity decreased by ~85% with the first 10% water addition. Further increasing the water content significantly increased IC to its maximum at 60 wt% water addition. This is attributed to a combined effect of viscosity reduction and increased ion mobility, which results from promoting ionic dissociation in DES. However, IC started to decrease when the water content was increased beyond 60 wt% due to the dilution effect of electrolytes. Similar results were reported for 1,2-propanediol: choline chloride: water (1:1:1) (Dai et al., 2015; Pierucci et al., 2018), glucose: choline chloride: water (2:5:5), and sucrose: choline chloride: water DES systems (1:4:4) (Dai et al., 2015). However, the viscosity gradually decreased after 10 wt% water substitution [Figure 6.1 (d)]. The high viscosity restricts the mobility of the free species within the DES matrix; thereby, the addition of water expands their applications in the industry. The density of most pure DES ranges from 1.1 to 1.2 g/cm³ (Manasi et al., 2021; Zhu et al., 2016), which is comparable to our results, as shown in Figure 6.1(b). The pH is a crucial physical property that significantly impacts chemical reactions. The pure DES (0 wt% water) exhibited a slightly acidic condition (5.72 ± 0.18), as shown in Figure 6.1 (c), which agreed with previously reported data for the ChCl/Gly DES system (Skulcova et al., 2018). This acidic pH primarily resulted from the alcohol structure of the glycerol (HBD), and it has been reported that HBD has a significant effect on determining the pH of the DES system (Skulcova et al., 2018). According to Figure 6.1 (c), it was observed that the addition of water resulted in a significant drop in pH from 5.72 to 4.26 at 40 wt% water

content and, then again, it increased gradually with the increasing water level, probably due to dual act of water (as both HBD and HBA). Similar trends in these physical properties were reported for the reline (Shah & Mjalli, 2014a) and various glycol-based DES systems (Gabriele et al., 2019).

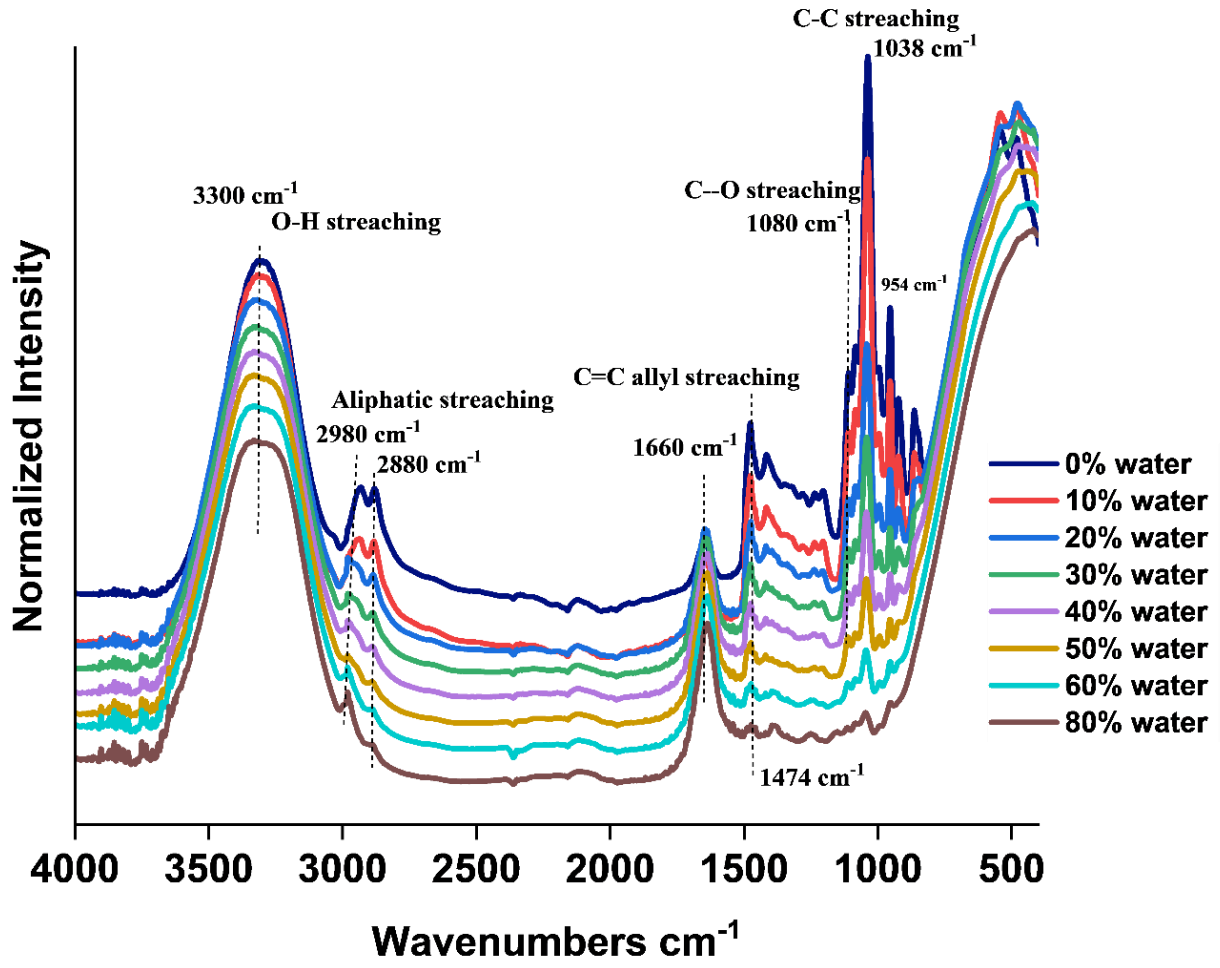


Figure 6. 2. Normalized FTIR spectra of the ChCl/Gly DES system (1:2 molar ratio) at varying water contents (0-80 wt%).

The structural changes and interaction of various functional groups at different levels of water contents are shown in Figure 6.2. The DES IR spectra generally have two distinct regions: the high wavenumber region (HWR) (4000–2500 cm^{-1}) and the fingerprint region (FFR) (1800–900 cm^{-1}) (Elderderi et al., 2020). In HWR, a broad band was observed for all samples from 3000-

3500 cm^{-1}, characteristic of O-H stretching vibration, which is associated with several HBs between HBD (Gly) and HBA (ChCl). Increasing the water content from 0 to 80 wt% considerably broadens the O-H stretching vibration peak with greater intensity, reflecting the contribution of O-H stretching vibrations from the water molecules (Alomar et al., 2016). Computational studies have suggested that the O-H stretching reflects multiple types of hydrogen bonds, including $OH(HBD)\cdot Cl^-$ and $OH(ChCl)\cdot Cl^-$ (Perkins et al., 2014). The low-intensity bands between 2921-2880 cm^{-1} (2980 and 2880 cm^{-1}) correspond to the C-H stretching vibrations of choline and glycerol, respectively (Zhu et al., 2016). With water content above 10 wt%, the band at 2980 cm^{-1} exhibits a shift (blueshift) in all DESs, while the peak at 2880 cm^{-1} nearly disappears after 40 wt% water addition. This suggests that the ChCl/Gly DES system with 10 wt% water is a transition point, altering intermolecular interactions and molecular arrangement.

As in Figure 6.2, the FFR region is also strongly affected by water addition. The peaks detected at 1038 and 1080 cm^{-1} belong to C-C and CH_2-CH_2-O stretching in the choline structure (Zhu et al., 2016). DES with 0 and 10 wt% water content showed an intense peak at 1030 cm^{-1}; however, the peaks became less pronounced with further addition of water (>10 wt%). Therefore, as discussed earlier, a DES with 10 wt% hydration is a critical transition point for assessing interactions between DES components, protein behavior, and molecular dynamics. The last strong peak between 964-939 cm^{-1} (~954 cm^{-1}) corresponds to the C−C stretching vibration and the NC_4 asymmetrical stretching vibrations. This was in line with the reported data, as the peak near 954 cm^{-1} is associated with the ammonium structure in ChCl (Aissaoui, 2015).

NMR Spectroscopy was used to characterize the impact of water on the supramolecular structures of the ChCl/Gly (1:2) system. The water content of pure DES was determined at 25 °C as water activity, since choline chloride is hygroscopic and the solvent can absorb water from the

surrounding atmosphere during preparation. The water activity of pure DES was found to be 0.028 ± 0.001, which we considered the DES with no added water (0 wt%). Since this is residual water confined in pure DES, it may impact the nanostructural organization of DES. The ^{1}H NMR spectra and the effect of water content on the ^{1}H chemical shift of DES are presented in Figures 6.3 and 6.2S (Appendix 16). The resonance at 4.25 ppm corresponds to the water in the DES system. The hydroxyl protons of ChCl (4c) and Gly (4g and 3g) showed distinct chemical shifts. This may indicate a slow exchange of hydroxyl protons, resulting from a strong HB network between DES components (Ferreira et al., 2021). It is seen that most signals have a downfield shift in the presence of water. This effect was more pronounced with the water and hydroxyl proton resonances (4c, 4g, and 3g), which completely disappeared after the addition of 30 wt% water, possibly due to the disruption of the internal HBs with the newly formed DES-water hydrogen bonds. The chemical exchange rate between hydroxyl and water protons increases when the DES mixture becomes richer in water. As a result, water and DES hydroxyl protons merge into a single signal resonance. This becomes apparent above the 30 wt% water addition and appears sharper at 40 wt%. Beyond this water composition, however, water protons experienced larger upfield shifts, which may signify the complete dissociation of the DES network. Moreover, the alkyl protons (1c, 1g, and 2c) show a larger shift for water content above 20 wt%. On the other hand, Aliphatic protons exhibited distinct chemical shifts even though they were not directly involved in HB interactions. It can be seen that the aliphatic group of ChCl, N-CH_3 (3c), was not affected at all within the entire dilution range studied, but aliphatic protons of glycerol (1g and 2g), and -CH_2OH (1c) and N-CH_2 (2c) of ChCl were not affected until the 10 wt% water addition. This suggests that the DES nanostructure is unlikely to be disrupted by the 10 wt% water in the DES matrix. Above 20 wt% water, however, a clear separation occurred, with downfield shifts in these protons, likely attributed

to the solvation effect. Our results align with those of Ferreira et al. (2021), who observed downfield shifts in the N-CH3 and N-CH2 protons at water contents above 40 wt%. They further evaluated the analysis of ^{35}Cl NMR and revealed that water has a restructuring effect on the DES, where water hydrates Cl^- anion first and then preferentially Gly and Choline, respectively. Moreover, Gabriele et al. (2019) investigated the effect of D_2O on the ChCl/glycol DES system and obtained results that were contrary to our study. They observed an upfield shift in the $N\text{-}CH_3$ and $N\text{-}CH_2$ protons of ChCl, while the CH_2OH group of ChCl and the glycol methylene remained largely unaffected by solvation. This behavior was attributed to hydrogen bonds between chloride and HBD, which shield the methyl and methylene protons.

Our results showed that water concentrations up to 10 wt% can occupy the free volume in the DES nanostructure with minimum disturbance to the network. Above this threshold, water starts to weaken some interactions while preserving the native structure up to a water content of 40 wt%. However, the DES system begins to behave as a solution of individual components at water additions exceeding 40 wt%.

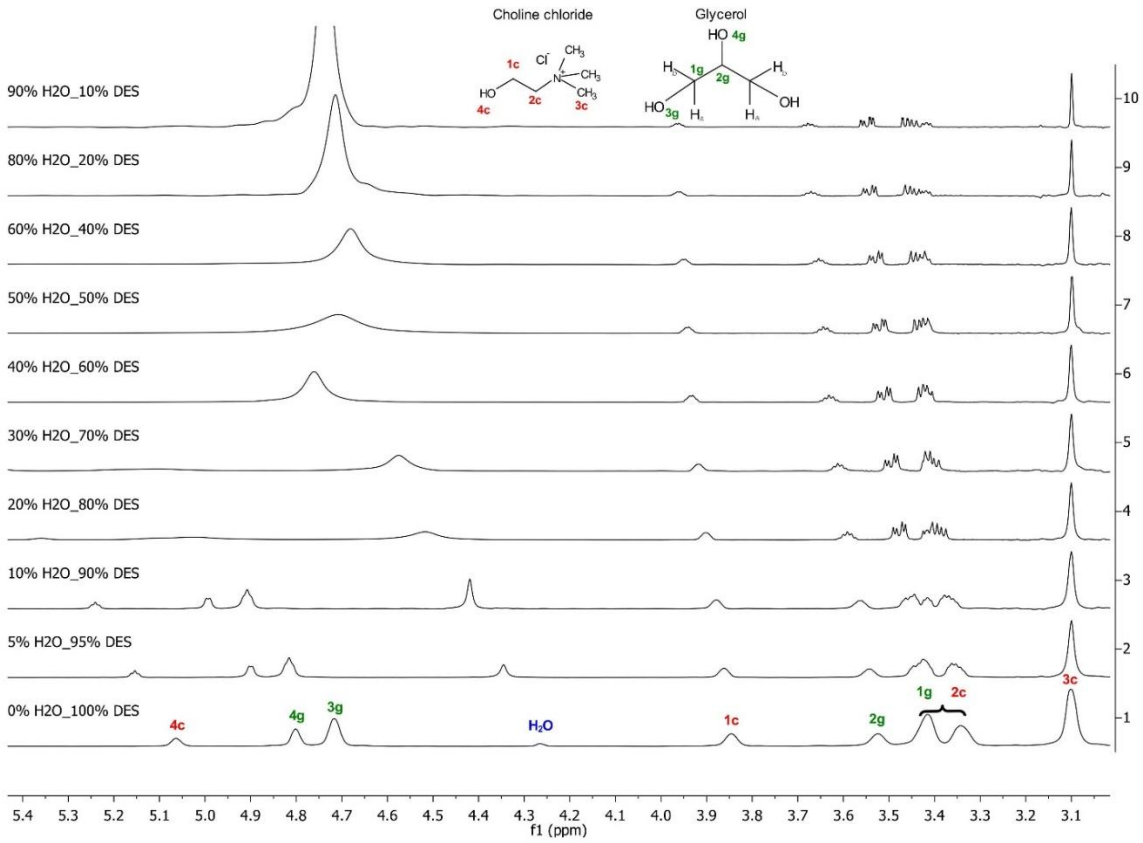


Figure 6. 3. ^{1}H NMR spectrum (600 MHz, C_6D_6 capillary as external reference) of choline chloride: glycerol (1:2) DES system with water content, 0-90 wt %.

6.4.2. Protein extraction using DES with different water substitution levels

Table 6. 2. Protein content, yield, and recovery rate of extracted proteins

Protein fraction extracted with DES/Water system	Protein content %	Protein yield (PY)%	Protein recovery rate (PRR) %
0FBP	86.38 ± 1.87^{c}	29.15 ± 3.13^{d}	10.29 ± 1.10^{d}
10FBP	95.33 ± 0.96^{a}	30.57 ± 0.15^{d}	10.79 ± 0.05^{d}
20FBP	92.19 ± 0.45^{ab}	37.55 ± 0.49^{cd}	13.25 ± 0.17^{cd}

30FBP	92.23 ± 1.14^{ab}	38.25 ± 1.30^{cd}	13.50 ± 0.46^{cd}
40FBP	91.82 ± 0.17^{b}	51.39 ± 1.29^{bc}	18.15 ± 0.45^{bc}
50FBP	89.65 ± 0.19b^{c}	55.69 ± 3.87^{b}	19.66 ± 1.36^{a}
60FBP	89.86 ± 2.30^{b}	77.12 ± 1.34^{a}	27.23 ± 0.47^{a}
80FBP	90.00 ± 0.94^{b}	78.76 ± 1.37^{a}	27.81 ± 0.48^{a}
100FBP	68.32 ± 0.74^{d}	83.83 ± 1.08^{a}	29.60 ± 0.38^{a}

All data were analyzed using analysis of variance (ANOVA) followed by Tukey's test (n=3) and expressed on a dry weight basis. Different superscripts within a column indicate a significant difference ($P < 0.05$). DES/water - Deep eutectic solvent composed of choline chloride and glycerol in a 1:2 molar ratio, with a varying water content ranging from 0 to 100 wt%. 0FBP-100FBP corresponds to fava bean protein extracted from water content 0-100 wt % in DES.

Adding water to DES significantly affected protein content, PY, and PRR, as indicated in Table 6.2. In the absence of water in DES (0FBP), both PY and PR were significantly lower ($P < 0.05$) compared to all other protein fractions obtained at different water levels in DES. The high viscosity of DES hinders the mass transfer and mobility of solutes within the extraction phase, thereby lowering PY and PRR. In particular, diluting DES with water up to 80 wt% led to an approximately 2.7-fold increase in PY and PRR. Initially, PY and PRR increased gradually within the 0-50 wt% water content range. Nevertheless, when DES was rich in water content at 60 wt%, both PY and PRR increased drastically, and then values increased at slower rates. Yue et al. (2021) demonstrated that a mixture of ChCl and butanediol isomers (1:3) with water binary mixtures performed better in protein extraction from oats than DES alone. In contrast, the effect of water content (10–90 wt%) in the ChCl/Gly DES system on soy protein extraction was found to be

reduced in efficiency when the water content exceeded 50–65 wt% (Chen et al., 2021). This was attributed to the disruption of DES and weakened interactions between the protein and DES, leading to decreased protein stability and solubility. In this study, the water extraction of protein was also carried out using the same extraction variables applied to the DES extraction. The pure water extraction resulted in significantly higher PY (83.83 ± 1.08%) and PRR (29.60 ± 0.38%) compared to DES extraction, but the protein content was significantly lower (68.32 ± 0.74%), possibly due to the higher carbohydrate content of the extractant.

Figure 6.4 illustrates how water addition affects the DES nanostructure, thereby potentially altering the intermolecular interactions between the DES and water, as well as the proteins. Experimentally, numerous studies have confirmed the distinct molecular-scale, three-dimensional intermolecular Hydrogen Bonding network of DES. Pure DES exists as similar to polymer structure (Liu et al., 2018b), where, in general, it appears as a self-assembled layered structure with a distinct ordering pattern between the DES components (Figure 6.4) In this study, the trace amount of water found in the pure ChCl/Gly DES system did not affect the supramolecular structure, as confirmed by the NMR analysis. At this point, the viscosity of DES is extremely high, significantly hindering the mass transfer rate during the extraction phase. Since hydrogen bonds are the primary driving force behind protein dissolution, the limited interaction of proteins with DES components may reduce solubility. When the water content is increased to 10 wt%, water molecules can insert themselves into the void spaces of the DES nanostructure without disrupting the HB network. Although we observed a drastic reduction in viscosity, the mobility of solutes within the DES matrix could still be hindered to a certain extent. Therefore, interactions between DES components and proteins might not be significant. Above 10 wt% water, noticeable structural and physical changes occur within the DES system, allowing proteins and water molecules to interact

significantly with DES components through hydrogen bonding, electrostatic interactions, and van der Waals forces. As a result, protein dissolution in the extraction phase increases, particularly above 50 wt% water, where the complete dissociation of DES components enables faster protein hydration. A higher water concentration in the DES system can break the strong HBs between glycerol and chloride ions by hydrating the chloride ions and forming HBs between glycerol and water. Therefore, adding water may disrupt the unique interaction between proteins and DES. This could cause the unfolding of proteins and, thus, changes in the protein's surface charges. These results showed that the tolerance limit for the microstructural transitional point from "water in DES" to "DES in water" was 40 wt%. In contrast, Ferreira et al. (2021) reported that 35 wt% water was the upper limit for the ChCl/Gly (1:2) DES system to maintain a water-in-DES mixture behavior, as determined by NMR analysis. Other experimental approaches, such as molecular dynamics (MD), have suggested that the reline-based DES structure is disrupted at water contents ranging from 25 to 41 wt% (Kumari et al., 2018; Shah & Mjalli, 2014b).

Research into protein and DES interactions is relatively scarce, and few researchers have investigated the interaction of proteins with DES using model proteins. For example, Parisse et al. (2023) conducted MD simulations on two proteins, hen egg-white lysozyme and the human VH antibody fragment HEL4, in a 20 wt% hydrated choline chloride–glycerol (1:2) system. They reported that DES specifically interacts with two tryptophan residues of lysozyme via choline ions, potentially enhancing protein–DES interactions and promoting protein crystallization. Moreover, solvation effect on the structure of a lipase protein in two DESs containing a protein destabilizer [choline : urea (1 : 2)] and stabilizer [choline : glycerol (1 : 2)] and their 1 : 1 aqueous solution using MD simulations were reported (Qiao et al., 2021). Results indicated that the lipase conformation remained folded in both DESs and aqueous solutions, and water molecules altered the

conformation of the lipase protein's active site. Furthermore, it was revealed that the hydration of DES affected the solvation shell of the lipase by reducing the protein-DES HB interactions (Qiao et al., 2021). The fundamental behavior of plant proteins in DES remains unexplored; therefore, systematic studies are essential to elucidate protein–DES interactions in hydrated systems. In this context, the current study serves as an initial benchmark, paving the way for future research to understand protein behavior in DES systems.

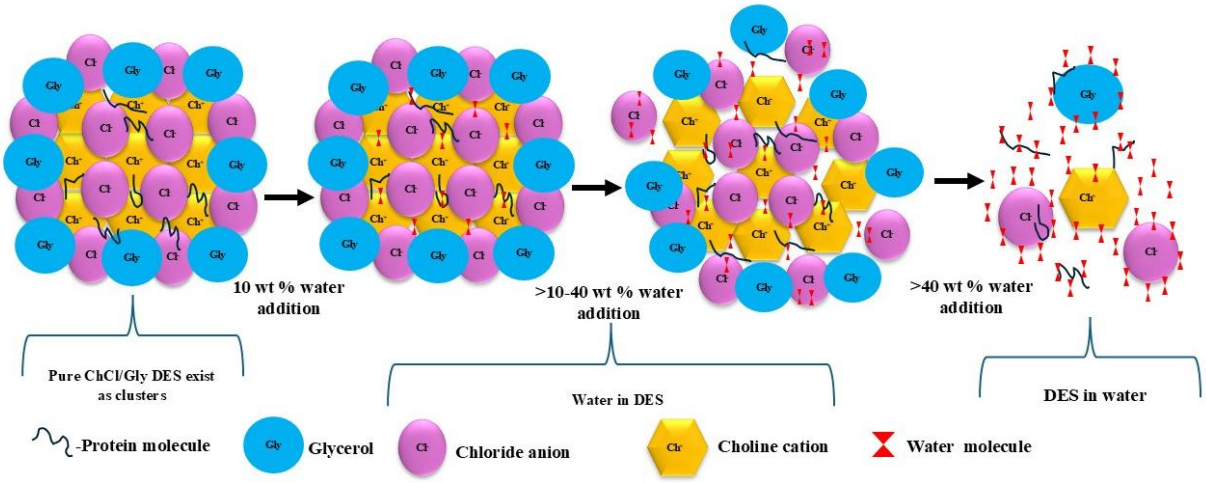


Figure 6. 4. The influence of hydration on the DES structure and mechanism in protein interactions. **(a)** pure ChCl/Gly DES system exists as clusters and resembles polymer structure, **(b)** 10 wt% water addition causes minimum distortion to the microstructure by inserting themselves into the void spaces of DES, **(c)** > 10 wt% water addition weakens the hydrogen bond network amplifying protein, molecules and water to interact with DES, (b&c) >10-40 wt% water addition considered as "water in DES" Hydrogen bond network is weakened. Still, it preserves the native structure of DES, **(d)** > 40 wt% "DES in water", complete dissociation of the DES structure.

6.4.3. Proximate composition

Table 6. 3. Proximate composition of extracted protein fractions

Protein fraction extracted with DES/Water system	Moisture %	Ash %	Fat %	Protein %	Total CHO %
0FBP	8.22 ± 1.35^{ab}	3.50 ± 0.84^{b}	6.66 ± 1.07^{a}	86.38 ± 1.87^{c}	3.46
10FBP	4.97 ± 0.69^{b}	2.58 ± 0.31^{b}	1.85 ± 1.06^{bc}	95.33 ± 0.96^{a}	0.24
20FBP	4.70 ± 0.95^{b}	2.22 ± 0.38^{b}	4.01 ± 0.32^{b}	92.19 ± 0.45^{ab}	1.58
30FBP	4.02 ± 0.27^{b}	2.45 ± 0.40^{b}	2.25 ± 1.58^{bc}	92.23 ± 1.14^{ab}	2.97
40FBP	4.35 ± 0.02^{b}	2.55 ± 1.11^{b}	1.60 ± 0.14^{c}	91.82 ± 0.17^{b}	4.01
50FBP	5.61 ± 2.48^{b}	2.58 ± 0.31^{b}	2.52 ± 0.84^{bc}	89.65 ± 0.19^{bc}	5.25
60FBP	10.36 ± 0.43^{a}	2.34 ± 0.14^{b}	1.85 ± 0.14^{bc}	89.86 ± 2.30^{b}	5.95
80FBP	9.93 ± 1.38^{a}	2.68 ± 0.12^{b}	2.82 ± 0.73^{bc}	90.00 ± 0.94^{b}	4.50
100FBP	10.75 ± 1.79^{a}	8.57 ± 0.12^{a}	0.85 ± 0.18^{c}	68.32 ± 0.74^{d}	22.26

All data were analyzed using analysis of variance (ANOVA) followed by Tukey's test (n=3) and expressed on a dry weight basis. Different superscripts within a column indicate a significant difference ($P < 0.05$). DES-Deep eutectic solvent containing choline chloride and glycerol with a 1:2 molar ratio from water content 0-100 wt%. 0FBP-100FBP corresponds to fava bean proteins extracted from water content 0-100 wt % in DES.

The proximate composition of extracted fava bean proteins (FBP) is presented in Table 6.3. Moisture content varied significantly across proteins obtained using different water levels in DES.

At 0 wt% water substitution (0FBP), the moisture content was significantly higher (8.22 ± 1.35%) than that extracted within the 10-50 wt% water range (10FBP-50FBP). However, 60FBP-100FBP showed significantly higher moisture content (9.93-10.75%) than all other FBPs produced. While pure DES is highly viscous, the dialysis process, performed over 5 days, may not have completely eliminated the solvent due to its strong bulk nanostructure. Therefore, the residual effect of DES may increase the moisture content of proteins. In contrast, when DES is rich in water (>50 wt%), DES components are fully hydrated and behave as solutes in aqueous solution (Bryant et al., 2021). Hence, hydrated DES could increase the possibility of higher protein solvation, subsequently affecting the higher moisture retained in the isolated protein fractions. Furthermore, the ash content of all counterparts was low, ranging from 2.2% to 3.5% ($P > 0.05$), except for the 100% water extractant, which showed a significantly higher amount (8.57 ± 0.12%). The observed differences could be related to the differences in the dialysis process, which was not performed for the pure water extraction. The fat content, 0FBP (6.66 ± 1.07%), had a significantly higher value than all other protein fractions. All the proteins extracted using the DES/water system exhibited a protein content of more than 89%, among which 0FBP had a significantly lower protein content (86.38 ± 1.87%). DES has been proven more effective in dissolving lignin and hemicellulose components (Gunny et al., 2015; Xu et al., 2016). This may be a reason for the lower purity in the 0FBP. However, with the addition of water, the solubility of lignocellulose materials decreases, allowing them to be removed efficiently through the protein recovery process. Therefore, at a water content of 10 wt% (10FBP), the protein content was significantly higher (95.33 ± 0.96%) than all other proteins extracted. DES (ChCl/Gly) with low viscosity and density, attributed to its superior diffusion properties and molecular mobility, was shown to extract a higher protein content in Canola (85.65%) (Karimi et al., 2024). Nonetheless, in our study, the protein content decreased

significantly when water content exceeded 10 wt%. It reached ~90% at an 80 wt% water content, likely due to the starch fraction of fava beans. This is evident in the results obtained for the pure water extraction of fava beans, where the protein content was significantly low (68.32 ± 0.74%) and a higher fraction of carbohydrates (CHO) was observed.

6.4.4. Physicochemical properties of protein isolate as impacted by water addition

6.4.4.1. Surface hydrophobicity

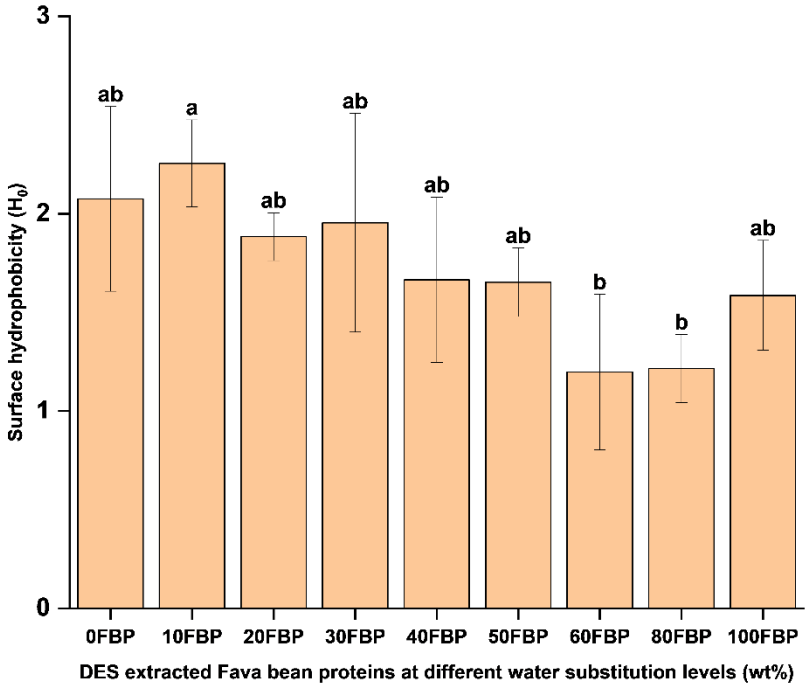


Figure 6. 5. Surface hydrophobicity (H_0) of fava bean proteins (pH 7.0) extracted using choline chloride: glycerol (1:2) DES system with water content 0-100 wt%. All data were analyzed using analysis of variance (ANOVA) and Tukey's test (n=3). Different letters indicate a significant difference ($P < 0.05$). 0FBP-100FBP corresponds to fava bean protein extracted from water content 0-100 wt % in DES.

Surface hydrophobicity is an important index used to characterize the protein's conformations and provide information about the extent to which the protein is unfolded, exposing its buried internal hydrophobic core (Jiang et al., 2015). The proportion of water in the DES system directly impacts the protein's conformations, as shown in Figure 6.5**.** Protein isolates obtained at the 10 wt% water content (10FBP) had a higher H_0 ($P < 0.05$) than all other protein isolates. This is likely associated with the partial or complete unfolding of proteins by the changes in DES and protein interactions (Figures 6.2S and 6.3). This is further evident with the increased percentage of β-sheets (Table 6.5) at the 10 wt% water addition. At lower hydration levels (10 wt%), the DES supramolecular structure begins to disrupt its native landscape; subsequently, it can induce alterations in the HBs and electrostatic interactions between protein and DES components (Sanchez-Fernandez & Jackson, 2016). This may lead to complex structural transitions in proteins. However, at increased hydration levels (20-50 wt%), these conformational changes may have allowed the proteins to retrieve their native conformation, as indicated by the significant reduction of H_0 in isolated proteins. Specifically, the Ho value was not significantly changed among the isolates obtained with this range of water content (20-50 wt%). It is explained that a DES system with a strong HB network between DES components may hinder the interaction of these components with proteins and water; however, the aqueous microenvironment formed by water induces the protein to fold into its native conformation (Sanchez-Fernandez & Jackson, 2016). Sanchez-Fernandez et al. (2017) reported that a hydrated (25 and 50 wt%) ChCl/Gly (1:2) system could retrieve the native conformation of the secondary and tertiary structure of bovine serum albumin (BSA). Similarly, the structure of lysozyme was preserved using hydrated DES compared to pure DES (Esquembre et al., 2013). The author noted that extensive dialysis during incorporation into pure DES restored the protein's native structure. Consistent with this

phenomenon, the current study observed that isolated proteins from pure DES and 100 wt% water systems exhibited similar behavior to that in DES with 20–50 wt% water content. This indicates that the pure ChCl/Gly DES system (1:2) can produce FBP with minimal denaturation, even at an extraction temperature of 50 °C, similar to that achieved with 100 wt% water extraction. Interestingly, if the water content increased (60-80 wt%), H_o decreased significantly to the lowest values among all isolates. This demonstrates that an aqueous solution of the discrete DES constituents may not affect the native conformation of the protein (Sanchez-Fernandez & Jackson, 2016).

6.4.4.2. Particle size distribution (PSD)

The volume-weighted mean particle diameter [D (4,3)] and surface area-weighted mean particle diameter D [(3,2)] of FBP aqueous dispersions are presented in Figure 6.1S **(**Appendix 15**)** and Table 6.4. In all cases, a relatively uniform monomodal distribution was observed for particle size diameters ranging from 0.5 to 350 μm, except in 100FBI, which flattened the size distribution curve to a bimodal distribution. The PSD measured for D (3,2) and D (4,3) varied significantly ($P > 0.05$) among the isolated proteins at different hydration levels in DES. The D (3,2) values reflect the fine particulates' size distribution, whereas D (4,3) provides the size of the coarse particulates. Moving from pure DES extraction to hydrated DES (up to 40 wt%) resulted in a successive reduction in average particle size; the same trend was observed after protein extraction was performed at water additions above 50 wt%. Specifically, the average particle size distribution of the isolates obtained from 10-40 wt% water content exhibited a significantly lower size than those extracted from DES with a water substitution above 50 wt%. Moreover, particle size was the highest at 50 wt% hydrated DES (50FBP) compared to those extracted with any other water substitution levels. In terms of fine particulates [D (3,2)], 100FBP had the lowest value (4.35 ±

0.08 μm) compared to all other isolates ($P < 0.05$). This may be attributed to the higher solubility of proteins (Yang et al., 2022). Considering the DES/water system, 20FBP (8.20 ± 0.08 μm) and 30FBP (7.43 ± 0.12 μm) had the lowest D (3,2), while 0FBP showed significantly higher distribution (32.93 ± 2.94 μm) with the same parameter. In the case of D (4,3), 50FBP exhibited a higher volume distribution ($P < 0.05$) compared to the other isolates. Based on our results, it can be explained that DES, without water, can produce protein clusters, which is simply an aggregated form due to the lower solubility of proteins, as there is limited interaction between DES components and proteins in a high-viscosity environment (Sanchez-Fernandez & Jackson, 2016). This could plausibly lead to higher particle sizes. When DES is mixed with water within its structural integrity, DES components and proteins remain well-coordinated through HBs, resulting in increased dispersibility (Dai et al., 2015; Sanchez-Fernandez & Jackson, 2016). Thus, particle sizes may become smaller. However, when the structural integrity of DES is disrupted (>40 wt%), proteins may aggregate due to the strengthening of the DES-water correlation. Additionally, water can form a strong hydration layer around the protein surface in this environment, thereby enhancing its solubility. Therefore, the particle size decreases further with the addition of more water. This may indicate that the FBP was produced using different hydration levels in DES, which affected the size of the protein particles.

Table 6. 4. Particle size distribution and thermal properties of extracted proteins

Protein fraction extracted with DES/Water system	Thermal properties			Particle size distribution (μm)	
	T_o (°C)	**T_d (°C)**	**ΔH (J/g)**	**D (3,2)**	**D (4,3)**
0FBP	67.31± 0.93^{a}	108.83± 6.27^{a}	86.24± 3.93^{g}	37.67 ±0.58^{a}	73.55± 1.67^{b}
10FBP	57.84± 1.77^{b}	103.64± 2.96^{a}	180.71± 1.97^{cd}	15.87± 0.23^{d}	40.48± 0.70^{c}
20FBP	55.2 ± 0.52^{b}	103.94± 0.71^{a}	124.16± 2.42^{f}	8.20 ± 0.08^{ef}	18.31 ± 0.32^{e}
30FBP	55.9 ± 0.86^{b}	104.90± 0.23^{a}	174.87± 3.92^{d}	7.43 ± 0.12^{ef}	17.79 ± 0.43^{e}
40FBP	57.7 ± 0.32^{b}	104.63± 0.47^{a}	183.82± 2.11^{bcd}	9.76 ± 0.14^{e}	21.54 ±0.36^{de}
50FBP	57.80± 1.54^{b}	102.55± 3.54^{a}	186.58± 1.84^{bc}	32.93± 2.94^{b}	95.41 ± 8.72^{a}
60FBP	56.84± 1.09^{b}	99.64 ± 1.16^{a}	222.95± 1.66^{a}	24.10± 0.01^{c}	67.69 ± 0.54^{b}
80FBP	57.11± 4.33^{b}	104.11± 3.89^{a}	194.57± 3.86^{b}	21.53± 0.54^{c}	71.65 ± 2.86^{b}
100FBP	56.04± 1.71^{b}	107.5 ± 1.86^{a}	153.52± 0.76^{e}	4.35 ± 0.08^{f}	33.90 ±0.99^{cd}

Thermal properties; T_o – onset temperature, T_d- peak transition temperature, ΔH- enthalpy, and particle size distribution; D [4,3]: Volume-weighted mean particle diameter, D [3,2]: Surface area-weighted mean particle diameter of FBP (fava bean proteins) extracted from different water content; 0FBP-100FBP- DES system with 0-100 wt% water content extracted fava bean proteins, All data were analyzed by analysis of variance followed by Tukey's range test (n=3). Different superscripts within a column indicate a significant difference ($P < 0.05$).

6.4.4.3. Effect of water addition to the DES system on the protein profile of extracted isolates

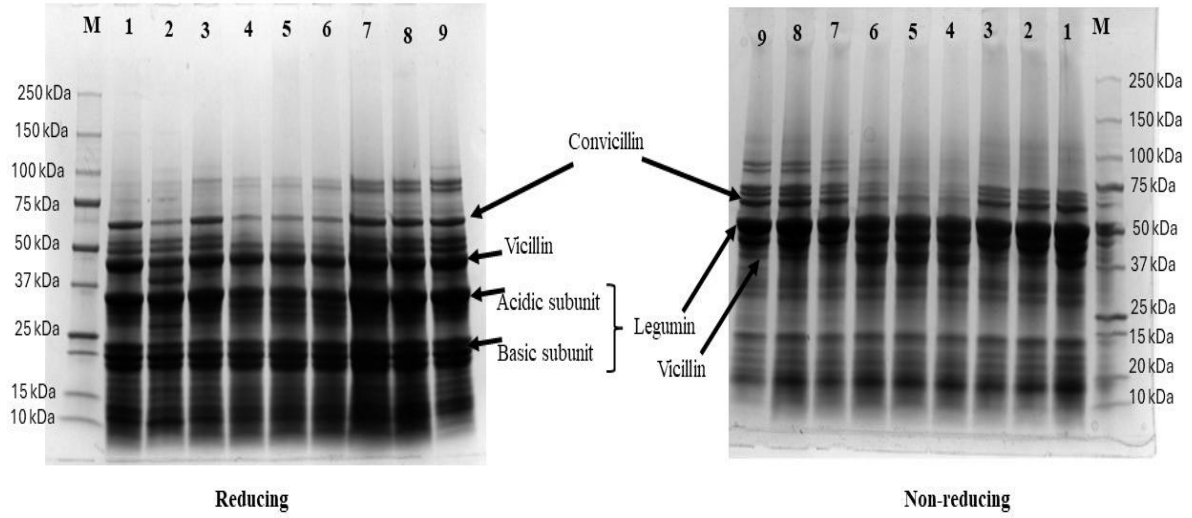


Figure 6. 6. SDS-PAGE profile of proteins extracted from ChCl/gly DES system (1:2) with different water content (0-100 wt%). Lane 1- 0% water DES system, Lane 2-, 10% water DES system, Lane 3- 20% water DES system, Lane 4- 30% water DES system, Lane 5- 40% water DES system, Lane 6- 50% water DES system, Lane 7- 60% water DES system, Lane 8- 80% water DES system, Lane 9- 100% water DES system, and Lane M- molecular weight marker.

Globulin is the primary storage protein in fava beans, classified into 11S proteins (legumin) and 7S proteins (vicilin and convicilin). Legumin is a hexameric holo-protein comprising three acidic (α chains, 40 kDa) and three basic (β chains, 20 kDa) monomers linked by disulfide bonds. Vicilin and convicilin, on the other hand, are trimers consisting of 50 kDa and 70 kDa subunits, respectively, held together by hydrophobic interactions and lacking cysteine units (Sharan et al., 2021). The SDS-PAGE was performed under non-reducing and reducing conditions for FBP, as depicted in Figure 6.6. FBP extracted with different water levels in the DES system showed differences in molecular weight distribution. Under non-reducing conditions, the SDS-PAGE

pattern of all FBP corresponded to convicilin (~70 kDa), legumin (~50 kDa), and vicilin (~37 kDa) (Vogelsang-O'Dwyer et al., 2020; Żmudziński et al., 2021). FBP obtained from the 0-30 wt% and 60-100 wt% water ranges showed slight disintegration at the vicilin polypeptide band (~37 kDa), whereas bands detectable at ~70 kDa (convicilin) were almost faded for the FBP extracted at hydration levels of 30-50 wt%. It is worth noting that bands appeared at ~98 kDa, which is likely corresponding to lipoxygenase (Badjona et al., 2024), and almost disappeared in the range of 0-50 wt% water addition, indicating that DES extraction could successfully eliminate the lipoxygenase activity of proteins.

Under reducing conditions, the legumin was further dissociated into acidic (~35-28 kDa) and basic (23-18 kDa) subunits. These polypeptide bands were much stronger in the 60-100 wt% water range than in other water ranges studied. Similarly, vicilin (~40 kDa) and convicilin (~70 kDa) also showed strong bands at the 60-100 wt% water range. Interestingly, as expected, with the 10 wt% water addition (lane 2) under reduced conditions, both vicilin and convicilin have slightly disintegrated, and two separate bands were detected at 30 and 37 kDa, which were not visible in the non-reducing conditions. Thus, these structural changes suggest that the DES system with a 10 wt% water content may tune the complex molecular interactions between the solvent and proteins.

6.4.4.4. Changes in protein secondary structures

Figure 6.3S (Appendix 17) illustrates the protein secondary structure components of the protein isolates. The FT-IR spectra of proteins represent four distinct bands, corresponding to β-sheets (1620-1635 cm^{-1}), α-helices (1650-1660 cm^{-1}), β-turns (1967-1969 cm^{-1}), and protein aggregates (1692-1696 cm^{-1}) (Shevkani et al., 2019). As shown in Table 6.5, substantial changes in secondary structure components were observed among the proteins extracted from the DES with different water content. 10FBP accounted for the highest percentage of β-sheets (32.22%) and the

lowest amount of α-helix (8.11%) among all isolated proteins. When transitioning from 0 wt% to 10 wt% hydration level, the amount of β-sheets increased by ~7%, while the α-helix decreased by ~5%. Further increasing the water content to 20 wt% resulted in a decrease in β-sheets from 32.22% to 26.54%, and this value remained constant until 50 wt% water addition. However, after adding water from 60 to 100 wt%, the contents of β-sheets increased to the same level as in the 20 wt% water substitution. Moreover, the α-helix content increased from 8.11% to 13.47% with the addition of 10 to 20 wt% water, respectively; however, beyond this range, a considerable change in α-helix content was not observed. In the case of β-turns, a decrease was observed when DES was rich in water (>50 wt%), even though no remarkable changes were noted in the range of 0-50 wt% water addition. Specifically, isolates produced from DES with a 10 wt% water content (10FBP) exhibited a small band at 1635 cm^{-1}, corresponding to β-sheets, which did not fully converge with the cumulative peak. This may be associated with protein unfolding. Since this structural change is not present in the spectra of other isolates, it can be attributed to the DES supramolecular structural changes induced by adding 10 wt% water, possibly directly correlating with the transition in protein conformation. These findings agree with those of Kumari et al. (2020), who reported changes in the secondary structure components of Lysozyme studied in pure and hydrated reline DES systems. Furthermore, a recent study demonstrated that the addition of water to ChCl/2,3-butanediol DES appreciably increased the α-helix content in oat proteins. Hydrated ChCl/1,2-butanediol and ChCl/1,4-butanediol, on the other hand, showed a decreased amount of α-helix (Yue et al., 2021). This suggests that water addition could alter the protein behavior even within the butanediol isomers. Therefore, the DES-water mixture is not simply an aqueous solution but a complex system that can change the protein conformation to various protein

structures. Thus, a fundamental understanding of plant protein behavior in DES for their conformational changes should be deeply investigated.

Table 6. 5. The relative proportions of secondary structure components of extracted proteins

Protein fraction extracted with DES/Water system	β-sheets %	α-helix %	β-turns %	Protein aggregates %
0FBP	25.29	13.90	14.17	6.27
10FBP	32.22	8.11	11.16	6.23
20FBP	26.54	13.47	10.80	ND
30FBP	25.04	13.28	14.27	6.49
40FBP	24.90	13.27	14.18	6.45
50FBP	24.92	13.27	14.20	6.46
60FBP	29.64	12.59	10.43	7.19
80FBP	31.14	13.43	8.43	6.60
100FBP	31.46	11.76	10.14	6.98

Secondary structure components analyzed from amide-I peak for deep eutectic solvent (DES) extracted fava bean proteins at different water content (0FBP-100FBP). Secondary structure components % were presented as average spectral analysis (n=3).

6.4.4.5. Thermal stability

Thermal stability is one of the key parameters related to protein function in food systems. When proteins are heated, their structure and chemical composition can undergo substantial changes, which is often observed in the food processing chain. This can alter the end product's functional and nutritional properties; therefore, thermal stability information is very useful in

determining the optimal processing conditions for protein foods. Hence, higher thermal stability can extend the protein's processing time (Feng et al., 2021). It is clear from Table 6.4 that 0FBP showed a significantly higher T_o value (onset temperature) of 67.31 °C compared to the other isolates, among which no notable differences were observed (~56-58 °C). However, in the case of the peak denaturation temperature (T_d), no significant differences were observed among the isolates ($P > 0.05$). Despite that, 0FBP had the highest thermal stability (108.83 °C). By continuing the water addition, the T_d value decreased at 10FBP (103.64 °C) and became almost constant up to 40 wt% water addition. Thereafter, thermal stability decreased to the lowest (99.64 °C) with 60FBP. Upon further replacement of water from 80-100 wt% in the DES system, the isolated proteins exhibited an increasing trend in thermal stability. Studies have shown that the solvation network confers the folding ability of proteins in DES, sometimes with similar structural behavior to that of proteins in aqueous solutions (Monhemi et al., 2014; Sarkar et al., 2017). It is believed that such interactions, in some cases, are responsible for the distinct folding of the proteins compared to that of water, where proteins remain either partially folded or in a compact state (e.g., lysozyme in 1:2 ChCl/Gly) (Esquembre et al., 2013). Consequently, this could lead to increased protein thermal and physical stability.

Another parameter for analyzing thermal stability is the enthalpy change (ΔH), which provides information about the extent of ordered protein structures (Kudre et al., 2013). Moreover, the ΔH results from the balance between endothermic reactions (breakup of hydrogen bonds) and exothermic reactions (protein aggregation and breakup of hydrophobic interactions) (Privalov, 1979; Peyrano et al., 2016). According to Table 6.4 for ΔH results, it can be seen that enthalpy changes were significant ($P < 0.05$) among all isolates and exhibited an opposite trend to the T_d behavior. 0FBI exhibited the lowest enthalpy (86.24 J/g), while the highest ΔH was found with

60FBI. Considering the ΔH behavior among isolates, the enthalpy increased when extracted with hydrated DES at 10-60 wt% and subsequently decreased upon further substitution of water (80-100 wt%). These enthalpy differences are probably due to the various percentages of secondary structure components observed in isolated proteins (Table 6.5). It appears that the proteins interact with the three DES systems in distinctly different ways: when "DES no water" (the DES supramolecular structure remains intact), "water in DES" (the DES components are still intact), and "DES in water" (the DES components are completely disassociated). Hence, the interpretation of the thermal parameters of proteins is somewhat critical, as these changes may be associated with various factors, including the viscosity of the DES, hydrogen bonding interactions, Van der Waals forces, and electrostatic interactions between DES and proteins at different hydration levels. Therefore, further studies are warranted to compare the thermal stability of proteins under hydrated DES systems.

6.4.5. Effect of water addition to DES on protein functionalities

6.4.5.1. Protein solubility

Protein solubility is a key determinant of protein functionalities, such as emulsifying ability and foaming capacity. Thus, it can serve as a good indicator for incorporating protein ingredients into various food systems (Grossmann & McClements, 2023). The solubility of FBP, measured at four different pH levels (3.0, 5.0, 7.0, and 9.0), is presented in Figure 6.7(a). The solubility of all proteins was greatest at high acidic and alkaline conditions, and lowest at the isoelectric point (pH 4.0-5.0). The solubility profiles of all proteins measured across all pH ranges differed significantly ($P > 0.05$). At pH 3.0, 0FBP had significantly lower solubility (41.18%) than all other isolated proteins ($P < 0.05$). This is associated with the high viscosity of pure DES, where mass transfer is minimal within the extraction phase, affecting the solubility of proteins. As can be observed at pH

3.0, when water is replaced with DES, solubility increases drastically in 10FBP (84.66%) and 20FBP (79.77%), presumably due to reduced viscosity and an enhanced mass transfer rate. However, with the further addition of water to the extraction process (10-100 wt%), solubility at pH 3.0 generally showed a decreasing trend ($P < 0.05$). This could be attributed to the changes in the surface charges of proteins, which are accompanied by conformational changes induced by the water content in DES. In the case of pure water extraction (100FBP), the solubility was unexpectedly low (39.06%) at pH 3.0 ($P < 0.05$), similar to that of 0FBP. Although dialysis was not performed for protein recovery in the pure water extraction, naturally occurring minerals in fava beans (Table 6.3) may influence the solubility of proteins (Jeganathan et al., 2023). Nonetheless, when the pH was increased from 5.0 to 9.0, the solubility of 100FBP was the highest ($P < 0.05$) among all extracted proteins, probably due to the higher solubility of the albumin fractions. At the isoelectric point, the solubility of proteins ranged from ~11-34%, which was far below that in other pH ranges. This is because the protein-protein interactions become energetically more favorable, decreasing electrostatic repulsions and enhancing hydrophobic interactions, thus reducing protein solubility (Ma et al., 2022). The solubility of all proteins at pH 7.0 (~83-86%) and 9.0 (>88%) is comparatively higher than at pH 3.0 due to the ionization of acidic and neutral amino acids at high pH (Kumar et al., 2021). Except in 20FBP, the solubility of all proteins obtained from DES extraction at neutral pH was not significantly different ($P>0.05$). Similarly, in alkaline conditions, the solubility behavior of most proteins did not differ significantly ($P > 0.05$). It is reported that oat proteins extracted by 1,4-butanediol-based DES/water binary mixture had greatly improved solubility (Yue et al., 2021). Moreover, canola protein extracted using ChCl/Gly/water (1:2:1) was shown to have superior solubility at pH 3.0 and 5.0, with

solubilities of 68.61% and 39.05%, respectively, compared to ChCl/D-Sorbitol/water, ChCl/D-glucose/water, and ChCl/urea/water DES systems.

6.4.5.2. Foaming capacity and stability.

The effect of water addition in DES protein extraction on foaming capacity (FC) and foaming stability (FS) of FBP is presented in Figure 6.7 (b). A substantially higher ($P < 0.05$) FC was found for 80FBP and 100FBP with values of 136.67% and 130%, respectively. The generation of larger foam volume depends on the higher solubility of proteins, which promotes the faster diffusion rate at the air-water interface. Especially in 100FBI, the higher solubility of albumin, smaller molecular size, and faster adsorption could contribute to the higher foam volume (Yang et al., 2022). Additionally, a higher vicilin and convicilin content is correlated with a faster adsorption rate, which favors a higher foam volume (Shen et al., 2024). Moreover, FC of isolates obtained from the 0, 20, 30, 40, and 50 wt% water in the DES (~64-66%) had similar effects ($P > 0.05$) among each other but higher than ($P < 0.05$) that of isolates produced using 10 wt% and 60 wt% hydration levels. As evident in the SDS- PAGE findings (Figure 6.6) and secondary structure analysis (Table 6.5) in this study, it can be seen that protein conformational changes occurred at 10% and 60% water addition, had a significant impact on the FC, in which proteins may not preferentially adsorb at the interface faster rate. Interestingly, these structural changes promoted higher foaming stability ($P < 0.05$) compared to all other samples [Figure 6.7(b)], demonstrating an opposite trend to the foaming capacity (FC). This may be attributed to the formation of stiffer interfacial layers. Hence, this could reduce the rate of disproportionation and the probability of coalescence of air bubbles. one study has shown improved foaming properties of extracted proteins by DES in the presence of water. Yue et al. (2021) reported higher FC and FS for oat proteins extracted using ChCl /1,4-butanediol and ChCl /2,3-butanediol-based DES with a water binary

mixture at a molar ratio of 1:3:1, indicating that the solubility of protein is positively correlated with FC.

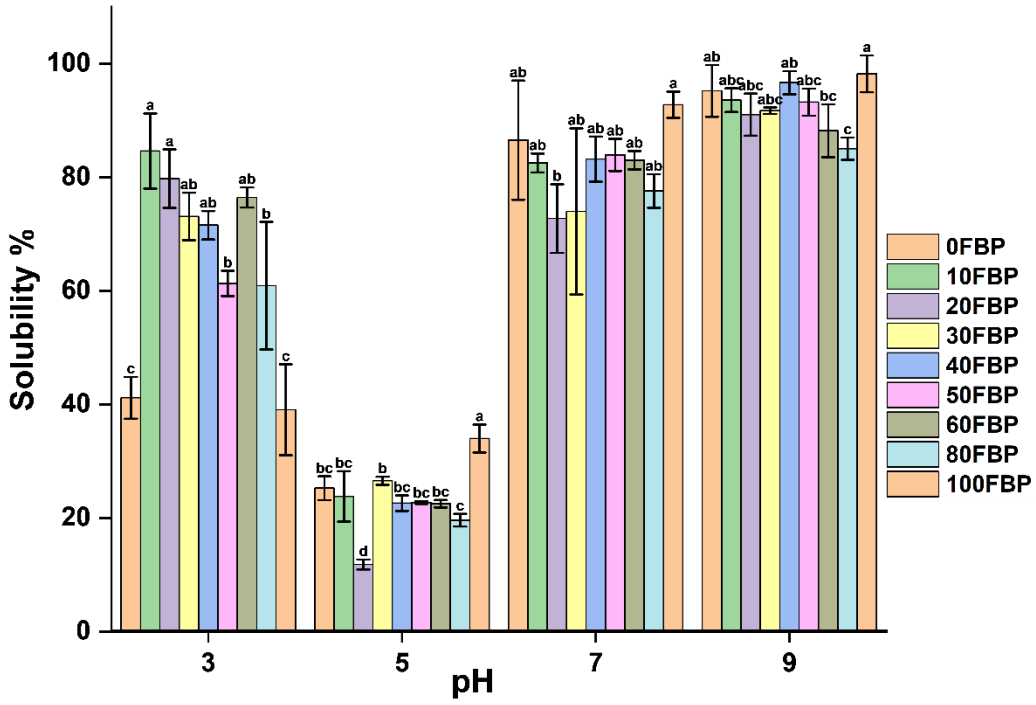
6.4.5.3. Emulsification properties

Emulsification properties are important in various food applications, including creams, sauces, desserts, beverages, ice cream, margarine, salad dressings, and soups. Protein ingredients are often used as emulsifiers due to their natural amphiphilic characteristics, interfacial activity, and colloidal stabilizing properties (Lima et al., 2023). The emulsion activity index (EAI) and the emulsion stability index (ESI) of FBP extracted with different water substitutions is illustrated in Figure 6.7(c). Among isolates, EAI ranged from ~43% to 53% under the tested conditions, and showed some similarities ($P > 0.05$) and differences ($P < 0.05$) among each other. Both 40FBP and 50FBP had almost similar EAI values, 52.38% and 51.41%, respectively, and were significantly greater ($P<0.05$) compared to all other isolated proteins. This suggests that these isolates were better able to unfold and adsorb to the oil-water interface by rearranging their conformation. The lowest EAI (43.27%) was with 100FBP, which may relate to their low molecular elasticity and/or low hydrophobicity (Zhang et al., 2023). All isolates followed this higher-to-lowest trend according to the EAI: 40FBP and 50FBP > 10FBP and 30FBP > 0FBP, 60FBP and 80FBP > 20FBP > 0FBP.

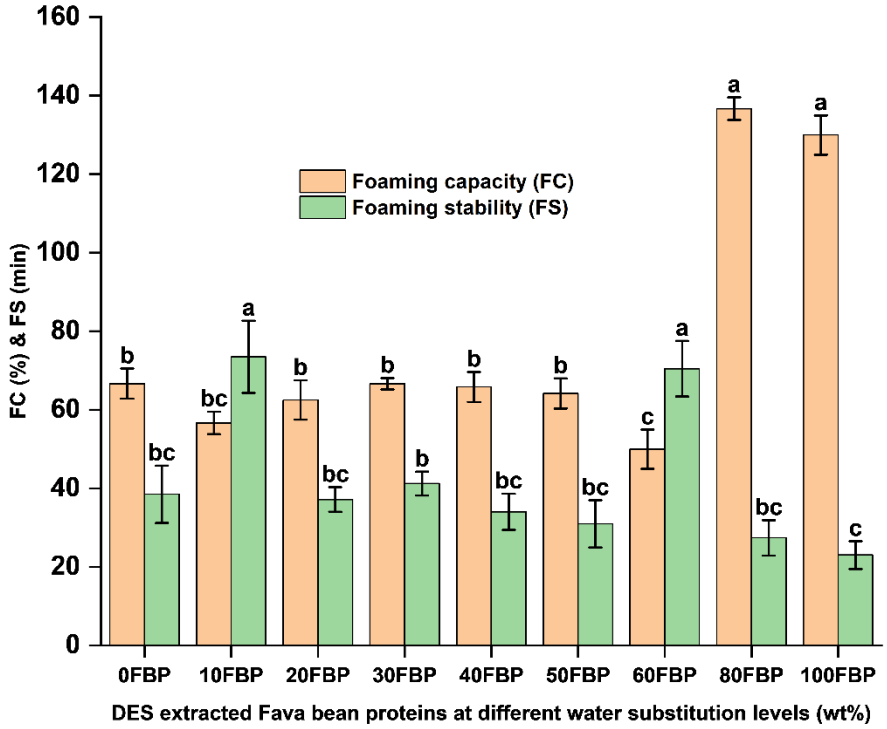
Emulsion stability refers to the extent to which the produced emulsion remains stable against droplet aggregation (Kumar et al., 2022). As shown in Figure 6.7(c), the EAI was comparatively low in all extracted proteins, ranging from ~11 to 13.5 min. Three isolates, 10FBP, 60FBP, and 80FBP, exhibited the highest stability compared to all other cases, but no significant difference was found among them. Furthermore, the lowest stability (~11 min) was shown by all 20FBP, 30FBP, and 50FBP. According to these results, it can be suggested that structural

differences induced by the DES/water interactions may play a critical role in determining the emulsification properties. We observed vast differences in the polypeptide bands corresponding to each isolate that appeared in SDS-PAGE (Figure 6.6), indicating that DES with varying water content could extract different proportions of protein subunits, legumin, vicilin, and convicilin. This might be associated with the differences observed in emulsification properties among the isolates. Additionally, hydrophobic residues exposed on the protein surface play a key role as the number and size of hydrophobic amino acids significantly correlate with protein solubility and aggregation states (Zhang et al., 2023). Thus, oil droplets can be stabilized by the proteins that accumulate on their surface, forming a strong viscoelastic film as a protective layer. In agreement with this, the differences in surface hydrophobicity of the extracted proteins observed in this study (Figure 6.5) may also contribute to the differences in emulsification properties. However, further investigations into the functional properties are needed at various pH levels and protein concentrations to explore a wide range of food applications.

(a)


Solubility %
pH
0FBP
10FBP
20FBP
30FBP
40FBP
50FBP
60FBP
80FBP
100FBP

(b)


Foaming capacity (FC)
Foaming stability (FS)
FC (%) & FS (min)
0FBP
10FBP
20FBP
30FBP
40FBP
50FBP
60FBP
80FBP
100FBP
DES extracted Fava bean proteins at different water substitution levels (wt%)

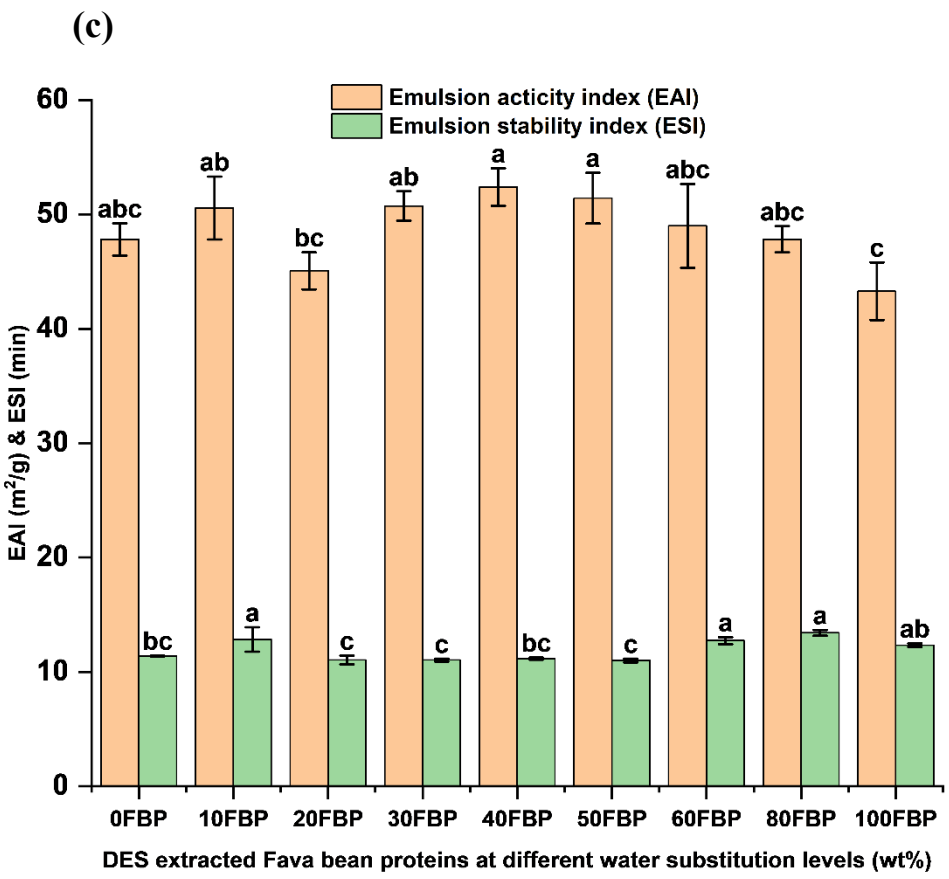


Figure 6. 7(a). Solubility % of extracted proteins as a function of pH, **(b)-**Foaming capacity (FC) and foaming stability (FS), and **(c)-** emulsification activity index (EAI) and stability index (ESI) of extracted proteins (pH 7.0 and protein concentration 1.0% w/v) at different water content in DES (0-100 wt%). All data were analyzed by analysis of variance, followed by Tukey's test (n=3). Different letters on the error bars indicate a significant difference at $P < 0.05$. 0FBP-100FBP corresponds to fava bean protein extracted from water content 0-100 wt % in DES.

6.5. Conclusion

This work evaluated the effect of water addition on the ChCl/Gly (1:2) DES system for the changes in the structure-functional properties of extracted fava bean proteins. One major barrier to DES applications is the high viscosity caused by a dense hydrogen bond network, which limits their industrial use. Diluting DES with water can help overcome this challenge by tailoring its properties for specific applications. This study demonstrates that adding 10 wt% water significantly reduces viscosity without altering the nanostructural organization of the DES, as evidenced by ^{1}H NMR and FTIR data. Based on the structural changes in DES brought by the addition of water, we identified three distinct behaviors: (1) 0-10 wt% water (water in DES) where structural integrity is fully maintained, (2) 10-40 wt% water (water in DES) where structural integrity is partially distorted and (3) >40 wt% water (DES in water) where the structure is completely distorted and behave as an aqueous solution. The extracted fava bean proteins clearly showed structural differences in their secondary structure components, and their thermal stability was identified within these three regimes. Interestingly, SDS-PAGE results showed distinct changes in the polypeptide bands following the addition of 10 wt% water. These structural alterations in proteins probably induced the changes in techno-functional properties. Therefore, the characteristic physiochemical behavior of proteins in DES is linked to the solvation effect. Additionally, the interaction of proteins with DES, particularly hydrogen bonding and electrostatic interactions, can significantly impact the structure and dynamics of the proteins. Since we recovered the protein through a rigorous dialysis process in this study, attention should likely be given to investigating and comparing whether the structure and dynamics of the protein are similar to those in the ChCl/Gly/water DES aqueous system. Hence, a fundamental understanding of protein behavior in DES provides a potential drive for designing novel DES systems for specific food applications.

Connection to Chapter 7

Based on the results obtained from Chapters 3, 4, 5, and 6, it is overall evident that DES protein extraction from fava bean can produce a protein yield of more than 60% and a protein content of 90%, similar to that of alkaline extraction. Industrially, alkaline extraction typically yields approximately 50% of the protein. Therefore, DES has greater potential in industrial applications. Due to DES-extracted fava bean's improved functionality and high protein quality, it can be further positioned for novel plant-based food applications. Notably, adding water to the DES system enables it to be transformed into a designer system, which can be utilized to develop specific functional properties tailored to a particular food application. Considering the plant protein ingredient market, soybean protein plays a dominant role over other plant proteins due to its superior nutrient quality and functionality. Thus, soybean protein is used as a benchmark for comparing those produced from other plant sources. However, concerns about allergens in soybean protein still pose a significant challenge to developing novel food products. In response to a market seeking alternatives to soy proteins, Fava bean protein, on the other hand, shows greater potential as a replacement for soybean protein, owing to its improved nutritional and functional attributes, as demonstrated in this research study. As observed in previous chapters, the superior gelling capacity, high protein content, and improved protein quality of DES-extracted fava bean proteins led to an in-depth investigation of gelling behavior, molecular interactions, and the gelling mechanism in Chapter 7. From a food application perspective, deep diving into the fundamental aspects of protein surface characteristics, gel formation, rheological properties, and molecular interactions in gel formation is crucial. This enables the food industry to utilize fava bean and scale its product offerings and operations with a range of innovative approaches for sustainable food options while building a resilient supply chain in Canada, including overseas.

Chapter 7

Understanding the fava bean protein gelation and its mechanism: Effect of novel deep eutectic solvents (DES) and conventional extraction methods on protein gelation.

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7.1. Abstract

The fundamental understanding of the gelation behaviour of DES-extracted fava bean protein isolates (DES-FBPI) is currently limited and has not yet been studied. We investigated the effect of choline chloride and glycerol-based protein extraction on the heat-induced gelation properties of fava beans, comparing it to conventional extraction methods: alkaline extraction (AE), salt extraction (SE), and commercial soy protein isolates (CS-PI), which served as a standard. DES-FBPI formed stronger, firmer gels (20% w/v protein, pH 7.0) than gels produced from proteins prepared using other isolation techniques. Gels from DES-FBPI showed a significantly higher ($P < 0.05$) final storage modulus (G'), gel hardness, and lower tan (δ) value (G" / G') compared to other gels. Overall, all gels were predominantly stabilized by hydrophobic interactions. However, gels made from DES-FBPI had significantly higher ($P<0.05$) disulfide bond content than gels made from other protein isolates, indicating a stronger gel network. The gel morphology of DES-FBPI gels exhibited a homogeneous, well-connected, fine-stranded, polymer-like structure. In contrast, gels made from alkaline-extracted fav bean protein isolates (ALK-FBPI) and salt-extracted fava bean protein isolates (SS-FBPI) consisted of irregular protein aggregates loosely stacked with larger pores, suggesting the formation of a particulate gel structure with weak intermolecular interactions. Gels produced from CS-PI, on the other hand, exhibited dense, heterogeneous aggregates of considerably larger size. In conclusion, this study demonstrates that DES extraction can modulate protein composition, promoting strong gel formation and enhancing the potential of various fava bean protein-based food formulations.

7.2. Introduction

Fava beans (*Vicia faba L.*) are an important source of protein and a promising sustainable alternative to animal-based foods in the human diet. They are rich in proteins (20-35%) with a high lysine content (Gulzar et al., 2024). Fava beans have a lower lipid content (<2%) and no cholesterol, serving as a versatile source of vegetable protein (Millar et al., 2019). The market expansion for the fava bean reached USD 3.18 billion in 2021 and is projected to increase to USD 3.47 billion by 2025 (Faba Beans Global Market Report, 2021). This primarily reflects the growing demand for plant-based proteins by consumers. Thus, the food industry has focused on producing novel protein ingredients and further processing them to meet the growing demand. The gelation properties are a crucial functional attribute of legume proteins and play a vital role in food processing and product formulations, such as tofu, tempeh, yogurt, cheese, and meat analogs, as food texture development primarily stems from the gel network formed by these proteins. Conventionally, protein gelation is achieved by heating, although some physical and chemical processes are also employed to induce gelation. Heat-induced gelation is a multi-step process that requires unfolding polypeptides, exposing the interaction sites, and aggregating protein molecules to form a three-dimensional gel network (Mengozzi et al., 2024). Hence, gel-forming ability and viscoelastic properties of proteins largely depend on intermolecular interactions, including covalent disulfide bonds (S-S), hydrogen bonds, and hydrophobic interactions. (Ge et al., 2023; Nicolai & Chassenieux, 2019). Understanding these interactions is crucial to modifying or controlling the textural properties of foods. Although heat-induced gelation profoundly varies among legume proteins, fava bean proteins have been shown to have comparable textural properties to soy proteins (Cai et al., 2001), which is often used as a benchmark to compare other legume protein gels due to its excellent gelling and structuring behavior (Banerjee & Bhattacharya, 2012; Berghout et al., 2015). However, there

is a strong interest in diversifying plant protein sources such as fava beans in view of their non-allergenicity and sustainability.

The major storage protein in fava beans is globulin, which accounts for 70–80% of the total protein. Globulin is a salt-soluble fraction composed of two main components: legumin (11S) and vicilin/convicilin (7S). Legumin has a hexameric structure with subunits of 50–60 kDa, each consisting of acidic (~40 kDa) and basic (~20 kDa) polypeptides linked by disulfide bonds. In contrast, 7S exists as trimers with 40–70 kDa subunits held together by hydrophobic interactions (Johansson et al., 2023; Nivala et al., 2021a). Due to the aforementioned structural differences, these protein fractions exhibit distinct behavior in gelation (Johansson et al., 2023; Oluwajuyitan & Aluko, 2024). Moreover, protein extraction parameters, such as the type of solvents, temperature, and pH, determine the composition (11S/7S ratio) of the resulting protein isolates (11S/7S ratio), thereby altering their gelling characteristics (Langton et al., 2020). The most common method to isolate proteins from fava beans is the alkaline method (AE), followed by isoelectric precipitation (Alavi et al., 2021; Eckert et al., 2019; Langton et al., 2020). Vogelsang-O'Dwyer et al. (2020) demonstrated that dry fractionated fava bean protein-rich flour (FPR) can form stronger gels than FBPI produced by acid extraction and isoelectric precipitation. The author suggested that higher solubility and solid content might have contributed to the higher gelling ability of FPR. In another study, the rheological properties and microstructure of heat-induced fava bean gels were compared between alkaline and soak extraction methods at acidic and neutral pH (Langton et al., 2020). Extraction methods did not affect the gelation properties; however, heat treatment resulted in the formation of particulate or fine gel structures at pH 5.0 and 7.0, respectively (Langton et al., 2022). Although AE is cheaper and more efficient in protein extraction, the key question remains how to obtain better-quality proteins. It is widely agreed that commercial production of protein isolates

results in their innate functionality of proteins to be lost during alkaline solubilization, isoelectric precipitation, and heating (Hewage et al., 2022). These harsh conditions lead to protein denaturation and aggregation, thus poor functionality. Therefore, alternative, sustainable, eco-friendly techniques are in great demand in the food industry for novel applications.

Deep eutectic solvents (DESs) are a new class of solvents that gained tremendous attention in plant protein extraction due to their highly recognized sustainable characteristics (Hansen et al., 2021). Fundamentally, DES components are believed to be self-associated via intermolecular hydrogen bonds (HBs). However, electrostatic and van der Waals forces are also likely to be established (Hansen et al., 2021; Smith et al., 2014). Even though few investigations have been reported on DES protein extraction from major cereal and legume crops, for example, fava beans (Hewage et al., 2024), oats (Yue et al., 2021), soy (Chen et al., 2021) and canola (Karimi et al., 2024) a fundamental understanding of the interaction between plant protein and DES solvents is relatively scarce at a molecular level. None of the aforementioned studies investigated the gelling behavior and gelation mechanism of the extracted proteins as impacted by the extraction methods. Our previous work demonstrated that choline chloride (ChCl) and glycerol (Gly) (1:2 molar ratio) based DES-FBPI has a lower least gel concentration and produces firmer gels than conventionally extracted FBPI (Chapter 4). Nonetheless, understanding the mechanisms that govern the formation of gels by DES-FBPI and their molecular interactions have not been explored yet. Therefore, we hypothesize that DES-FPBI can produce firmer gels due to differences in protein composition, molecular structure, formation of aggregates, and their interactions compared to conventionally extracted FBPI. Therefore, this study aimed to evaluate the gelation process, rheological and microstructural properties, and to characterize molecular structure of fava bean protein gels made from the DES-extracted FBPI using the ChCl-Gly-based DES system, compared to conventional

isolation techniques, including AE, SE, and CS-PI. The understanding of the mechanisms underlying the gelling behaviour of DES-extracted fava bean proteins would provide valuable insights into further investigating novel food formulations from fava bean proteins.

7.3. Materials and Methodology

Dehulled dried fava beans (Snowbird variety) were purchased from Prairie Fava (Glenboro, MB, Canada), while CS-PI was purchased from Fisher Scientific (Ottawa, ON, Canada). Laemmli sample buffer, 2-mercaptoethanol, Precision Plus protein standard (10-250 kDa), Coomassie Blue R-250, and 4-15% Mini-PROTEAN TGX precast protein gels were purchased from Bio-Rad Laboratories Ltd. (Mississauga, ON, Canada). All other analytical grade chemicals and reagents were purchased from Fisher Scientific (Ottawa, ON, Canada).

7.3.1. Preparation of fava bean protein isolates (FBPI)

Dried fava beans were milled using a Prater-Sterling Impact Mill (M-21, Prater Industries, Bolingbrook, IL, USA.) equipped with a 0.2 mm screen. Based on our previous study (Chapter 4), three protein isolation techniques, DES extraction, AE, and SE, were used to extract proteins from milled fava beans (Sections 7.1S, 7.2S, and 7.3S, respectively, in Appendices 18, 19, and 20). In the case of DES extraction, it was performed with slight modifications based on our previous study (Hewage et al., 2024). These techniques underwent slight modifications. The protein content of DES-FBPI, ALK-FBPI, SE SS-FBPI, and CS-PI used for the gelation study was 91.82 ± 0.17%, 91.69 ± 1.02%, 92.08 ± 0.98%, and 89.67 ± 3.42%, respectively.

7.3.2. Characterization of protein molecular and physicochemical properties

7.3.2.1. Attenuated total reflectance Fourier-transformed infrared spectroscopy (ATR-FTIR)

FTIR analysis of protein samples was conducted with an INVENIO S (Bruker Scientific LLC, MA, USA) ATR-FTIR for secondary structure analysis. The generated Infrared (IR) spectra,

in the 400-4000 cm^{-1} range with a resolution of 4 cm^{-1} and 120 scans, were normalized and then subtracted from the baseline before undergoing second derivative analysis. The curve-fitting procedure was performed for the Amide I peak (1600-1700 cm^{-1}) to determine the secondary structure components using the Savitzky-Golay smoothing function (window size 9 and the Gaussian peak fitting function) in Origin 2024 software (OriginLab Corporation, MA, USA).

7.3.2.2. Intrinsic fluorescence of protein

The fluorescence spectra of protein samples were acquired using an FP-8350 spectrofluorometer (Jasco Corporation, Japan) equipped with a 10 cm path-length quartz microcuvette, according to the method described by Oluwajuyitan & Aluko (2024) with slight modifications. A protein stock solution (10 mg/mL) was dispersed in phosphate (pH 7.0) buffer and centrifuged at 9600 g for 10 min. The resulting supernatants were diluted to 1 μg/mL using the same buffer. The emission spectra of diluted solutions were recorded from 290 to 500 nm with an excitation wavelength of 280 nm (for tyrosine and tryptophan) and a bandwidth of 5 nm at 25 °C.

7.3.2.3. X-ray photoelectron spectroscopy (XPS)

Axis Ultra (Kratos Analytical Ltd, Manchester, UK) X-ray photoelectron spectrometer (XPS) was used to characterize protein isolates for their elemental composition and changes in surface functional groups according to their extraction methods. For the chemical composition, the samples were analyzed using monochromatic Al K-alpha X-rays (1486.6 eV), a pass energy of 160 eV for survey spectra, and 20 eV for high-res/elemental spectra, and a charge neutralizer at a charge balance of 2.5 eV. The spectra obtained for each element were recorded in the following sequence: C 1s, O 1s, N 1s, and S 2p. The peak fitting of C 1s, O 1s, N 1s, and S 2p for functional group

determination was performed using Casa XPS software (version 2.3.25PR 1.0, Casa Software Ltd, Teignmouth, UK), provided by the instrument manufacturer.

7.3.2.4. Free sulfhydryl (SH) group

The free sulfhydryl contents of protein isolates were measured using Ellman's assay (Ellman, 1959) according to the method outlined by Rahman & Lamsal (2023) with slight modifications. Briefly, 250 µL of protein solution (2.5 mg/mL) was added to 2.5 mL of reaction buffer (0.1 M phosphate buffer, pH 8.0, with 1 mM EDTA) containing 50 µL of Ellman's reagent solution (4 mg/mL). The mixture was then allowed to stand in the dark for 15 min at 25 °C, and the absorption was recorded at 412 nm using a UV-spectrophotometer (Genesys 150, Madison, USA) against the buffer as a blank. The free SH contents were calculated using absorbance and a molar extinction coefficient of 14,150 M^{-1} cm^{-1} for the reaction buffer system.

7.3.2.5. Sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE)

SDS-PAGE was performed under reducing (R) and non-reducing conditions (NR) according to the method described by Shevkani et al. (2015) with slight modifications. Protein samples (4 mg/mL) prepared in 5% sodium dodecyl sulfate (SDS) were mixed with Laemmli sample buffer, with or without 2-mercaptoethanol, to achieve a final protein concentration of 2 mg/mL. Then, the samples were heated to 90 °C for 5 min and cooled to room temperature. After that, 5 µL of sample and 10 µL of precision plus molecular weight standard were loaded to SDS-PAGE 4-15% gradient gels, respectively, and electrophoresis was run at a constant voltage of 150 V. Gels were stained for 1 hrs using 0.1% Coomassie brilliant blue R-250 in 20% (v/v) methanol and 10% (v/v) acetic acid and destained in 50% (v/v) methanol and 10% (v/v) acetic acid for overnight. The resulting protein bands were imaged using the Biorad ChemiDoc^{Tm} Imaging system (Bio-Rad Laboratories Inc., CA, USA).

7.3.2.6. Size Exclusion Chromatography (SEC)

The molecular weight distribution of protein isolates was determined using an AKTA purifier Fast Protein Liquid Chromatography (FPLC) equipped with a HiLoad 16/600, Superdex 200PG column (GE Healthcare, Montreal, PQ, Canada), according to the method described by Alashi et al. (2014) with modifications. Briefly, protein samples (20 mg/mL) were prepared in a 0.05 M sodium phosphate buffer containing 0.15 M NaCl (pH 7.2) and filtered through a 0.2 µm syringe filter. A 0.5 mL aliquot of the sample (20 mg/mL) was analyzed using the FPLC system, and proteins were separated using phosphate buffer (pH 7.2) at a flow rate of 0.5 mL/min. The column was calibrated with ferritin (440 kDa), aldolase (158 kDa), conalbumin (75 kDa), ovalbumin (44 kDa), carbonic anhydrase (29 kDa), ribonuclease (13.70 kDa), and aprotinin (6.50 kDa).

7.3.2.7. Amino acid (AA) composition

AA contents were estimated using the AOAC official method 982.30 (AOAC International, 2012). Most AAs were determined using acid hydrolysis (24 hrs, 6 N HCl). The performic acid oxidation, followed by acid hydrolysis, was performed for sulfur-containing AAs (AOAC Official Method 985.28). Tryptophan was analyzed by alkaline hydrolysis according to the official method outlined in the ISO protocol 13904:2005 (ISO, 2005). Consequently, Shimadzu ultra-high performance liquid chromatography (UPLC) system (Kyoto, Japan), equipped with a Waters AccQ C18 column (100 mm × 2.1 mm, 1.7 µm), was used to derivatize and separate the hydrolyzed amino acids. For quality control purposes, the NIST soy flour Standard was used as a reference (Reference Material 3234), and quantified AAs were expressed on a percentage by weight basis.

7.3.3. Gel preparation and gel characterization

7.3.3.1. Least gel concentration (LGC) for protein

LGC was measured at a protein concentration (PC) of 5-20% (w/v) and pH levels ranging from 3.0 to 9.0 for all protein isolates, as reported in our previous study (Chapter 4) (7.4S-Appendix 21). The gels prepared from DES-FBPI at 20% (w/v) PC and pH 7.0 produced stronger, firmer gels than those formed by ALK-FBPI and SS-FBPI. Therefore, 20% (w/v) PC at pH 7.0 was used for all protein isolates to investigate gelling and viscoelastic properties, molecular interactions, microscopy, and texture analysis.

7.3.3.2. Rheological properties of gel

Rheological properties of all protein suspensions prepared from each extraction method at pH 7.0 and a protein concentration of 20% (w/v) were studied using a Discovery HR-20 rheometer (TA Instruments, DE, USA) equipped with a parallel plate geometry (40 mm) at 1 mm gap. A temperature ramp test was conducted to study the viscoelastic properties of gels; the protein suspensions were heated from 25 °C to 90 °C at a rate of 2 °C/min, holding at 30 min, and cooled to 25 °C at 2 °C/min with the strain and frequency of 0.1% and 1 rad/s respectively. Following the temperature ramp test, a frequency sweep test was conducted to record the storage modulus (G') and loss modulus (G'') at oscillation frequencies ranging from 0.1 to 100 rad/s under a constant strain of 1%. All samples on the rheometer were covered with a solvent trap and heat-sealable silicon grease to prevent moisture loss.

7.3.3.3. Molecular interactions of gels

The intermolecular interactions of gels were determined by using five different protein denaturing solvents: 0.05 M NaCl (S1), 0.6 M NaCl (S2), 0.6 M NaCl+1.5 M Urea (S3), 0.6 M NaCl+8 M Urea (S4) and 0.6 M NaCl+8 M Urea+0.5 M β- Mercaptoethanol (S5) based on the

methods outlined by Rahman & Lamsal (2023) & Yang et al. (2020). Briefly, 1 g of prepared gels was dispersed in 10 mL of the above denaturing solutions and homogenized at 15,000 rpm for 2 min. Then, each protein dispersion was stirred for 1 hrs at 22 °C, followed by centrifugation (20,000 g, 15 min, 4 °C). The protein solubility of the resulting supernatant was determined using the Bradford assay, with bovine serum albumin (BSA) as a standard. The differences in protein solubility between each solution were used to calculate the relative percentages of ionic bonds (S2-S1), hydrogen bonds (S3-S2), hydrophobic interactions (S4-S3), and disulfide bonds (S5-S4).

7.3.3.4. Electron microscopy of gels

Protein gels prepared under sections 7.3.3.1 and 7.4S (Appendix 21) were examined using an electron microscope (Quanta 650 FEG, FEI Company, OR, USA) according to the procedure described by Opazo-Navarrete et al. (2018) with slight modifications. First, the gels were cut into 1 × 1 × 0.5 cm-sized pieces and fixed with 2.5 mL/100 mL glutaraldehyde, prepared in 0.1 mol/L phosphate buffer (pH 7.0), at room temperature (22 °C). Thereafter, each gel sample was dehydrated using a series of ethanol solutions with concentrations of 50, 70, 80, and 90%, for 15 min each, and finally in absolute ethanol overnight. Before analyzing the gel microstructure, the samples were air-dried for 2 hrs, followed by coating with gold-palladium (Au-Pd) conductive film. Microscopic images were obtained using the xT microscope control (Ver. 6.2.8, Quanta 650 FEG, FEI Company, OR, USA).

7.3.3.5. Texture analyses- Compression test for gel strength measurement

The protein gels obtained from DES extraction and CS-PI with protein concentration at 20% (w/v) and pH 7.0 were analyzed for compression strength using a texture analyzer as described by Moreno et al. (2020) with modifications. Protein gels were prepared according to the procedure described in sections 7.3.3.1 and 7.4S (Appendix 21) in a cylindrical shape

polypropylene tube (1 cm diameter). Gels were cut into 1 cm height, and a compression test was performed using a stable Micro System TA-XT texture analyzer at room temperature (22 °C) with a 100 N load cell. The gel hardness was determined by the peak force (N) measured during the first compression cycle using an acrylic cylindrical compression plate with an 18 mm diameter probe, and pre- and post-test speeds of 2 mm/sec and 1 mm/sec, respectively.

7.3.3.6. Water holding capacity (WHC) of gels

The WHC of gels was measured according to the method adopted by Zhang et al., (2022) with slight modifications. After preparing gels, as described in 7.3.3.1 and 7.4S (Appendix 21), the unbound water was drained. Then, the gel samples were centrifuged at 4000 g for 20 min, and the weight of the gel was recorded before and after centrifugation. The WHC of gels was calculated according to equation (1).

$$WHC = \frac{M_1 \times M_2}{M_1} \times 100 \quad (1)$$

Where M_1 represents the mass of total water in the gel before centrifugation (g), and M2 represents the mass of the gel after centrifugation (g).

7.3.4. Statistical Analysis

Statistical Analysis was performed by analysis of variance (ANOVA) at a 95% confidence level, followed by a Tukey test using MINITAB statistical software (MINITAB version 20, LLC, PA, USA) with a significance level of $P < 0.05$. All experiments were carried out in three replicates (n=3), except for Rheological analysis (n=2), and all results were presented as mean ± standard deviation.

7.4. Results and Discussion

7.4.1. Impact of different protein extraction methods on the molecular, physicochemical, and structural properties of fava bean protein isolates

7.4.2. Protein profile and composition

The protein pattern of protein fractions under non-reducing and reducing conditions is presented in Figure 7.1. Under non-reducing conditions, three major bands for all FBPIs were visible at ~65 kDa, ~53 kDa, and ~40 kDa, likely corresponding to convicilin, legumin, and vicilin, respectively (Vogelsang-O'Dwyer et al., 2020). Under reducing conditions, the legumin polypeptide band (~53 kDa) dissociated into its characteristic subunits, 30-35 kDa and 19–23 kDa, relating to the acidic subunit (α-legumin) and basic subunits (β-legumin), respectively (Żmudziński et al., 2021).

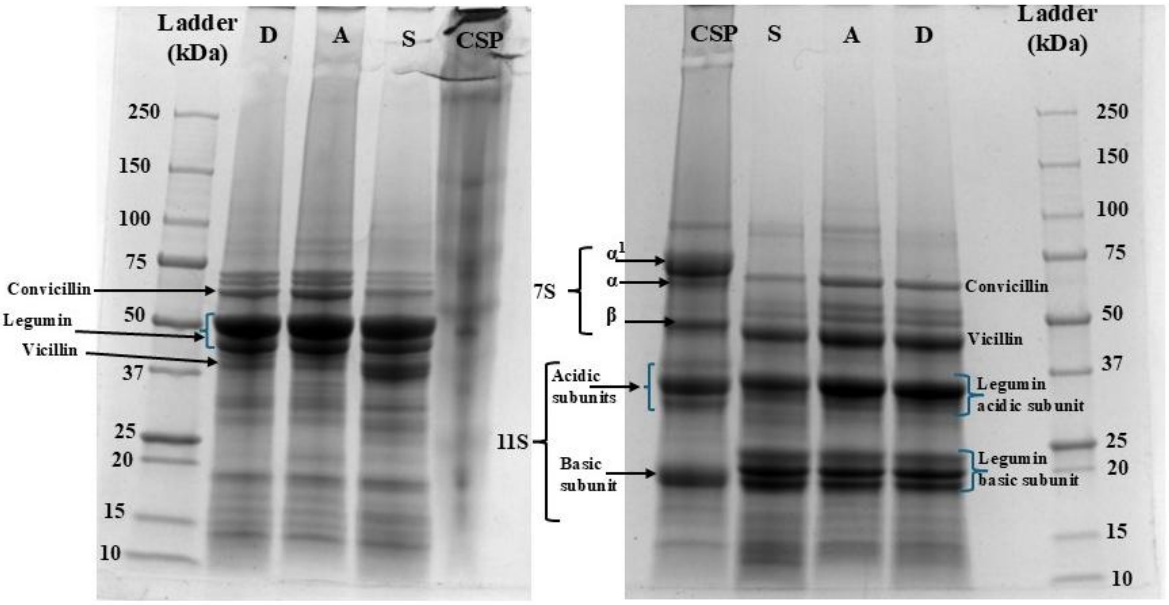


Figure 7. 1. SDS-PAGE profile of protein isolates obtained from various extraction methods, *i.e.,* Lane (D)- Deep eutectic solvent extraction, (A)- Alkaline extraction, and (S)- Salt extraction, and (CSP)- Commercial soy protein isolates, and Lane ladder- molecular weight marker (kDa).

A minor band at ~74 kDa was also detected, possibly attributed to minor legumin subunits (Gulzar et al., 2024). It is clear that under both conditions, the protein subunit, convicillin, and minor legumin subunits almost disappeared with SE compared to AE and DES extraction methods. However, under non-reducing conditions, the vicilin band was more pronounced in SS-FBPI, which was barely detected with DES-FBPI and ALK-FBPI. Moreover, a slight reduction in the intensity of both vicilin and legumin acidic subunits was observed for SE under reducing conditions. The albumin fraction of ALK-FBPI was disintegrated more than the DES and SE fractions, which agreed with our previous study (Hewage et al., 2024). Overall, SE and AE caused more prominent changes in FBPI than DES extraction, indicating partial degradation or fragmentation of protein subunits.

Compared to fava bean proteins, soy proteins have four major groups: 2S, 7S, 11S, and 15S fractions. The 2S (20% of the soy proteins) fraction contains most albumin fractions, whereas globulins consist of 7S, 11S, and 15S fractions. 7S globulin [40% of the soy proteins (β-conglycinin)] and 11S globulin [30% of the proteins (glycinin)] are the two major components in soy storage proteins. β-Conglycinin (~180 kDa) is a trimer composed of glycosylated subunits, α, α′, and β, held together by hydrophobic and electrostatic interactions. Conversely, Glycinin (~360 kDa) is a hexamer composed of acidic and basic subunits interlinked by disulfide bonds (Sui et al., 2021; Zheng et al., 2022). Based on the protein profile of CS-PI (Figure 7.1), the polypeptide bands were not visible under non-reducing conditions, which may be due to alterations in the protein caused by the commercial process. However, under reducing conditions, the protein bands of ~72

kDa, 63 kDa, and 49 kDa were identified as the α′, α, and β-subunits of the 7S globulin, respectively (Zhang et al., 2024). Three acidic subunits and a basic subunit of the 11S were detected at ~37 kDa, 35 kDa, and 32 kDa, and 20 kDa, respectively (Zhang et al., 2024). In addition, lipoxygenase (~ 100 kDa, less abundant proteins) appeared under reducing conditions. Nonetheless, two high-molecular-weight polypeptides were detected above ~250 kDa and ~150 kDa under non-reducing conditions. This could have been likely influenced by the high-temperature heat treatment processing of soy protein-induced aggregates via covalent and non-covalent forces, which explains why commercial processing produces abundant protein aggregates (Aryee et al., 2018).

7.4.2.1. Protein molecular weight distribution

Table 7. 1. Summary of the peak areas from the SEC profile at 214 nm of extracted proteins, as presented in Figure 2

Protein ingredients	Peak area %			
	11S	7S	Soluble aggregated protein fractions	11S/7S ratio
DES-FBPI	48.36	17.82	8.83	2.72
ALK-FBPI	30.08	20.36	16.07	1.48
SS-FBPI	32.62	14.02	9.80	2.33
CS-PI	7.98	13.48	41.11	0.59

Peak area (%) of size exclusion chromatography (SEC) profile of proteins extracted by various extraction methods, *i.e.,* Deep eutectic solvent-extracted fava bean protein isolates (DES-FBPI), Alkaline-extracted fava bean protein isolates (ALK-FBPI), Salt-extracted fava bean protein

isolates (SS-FBPI), and commercial soy protein isolates (CS-PI). 11S and 7S-protein subunits (S-Svedberg unit for sedimentation coefficient).

To further confirm the molecular weights (MWs) of the extracted proteins using different extraction methods, size-exclusion chromatography (SEC) was employed to reveal changes in the proteins' structural composition. The chromatographic results are presented in Figure 7.2. The FBPIs obtained from AE, SE, and DES showed apparent MWs in the range of 140-190 kDa and 87-91 kDa, attributed to legumin (11S) and vicilin/convicilin (7S), respectively (Yang et al., 2018).

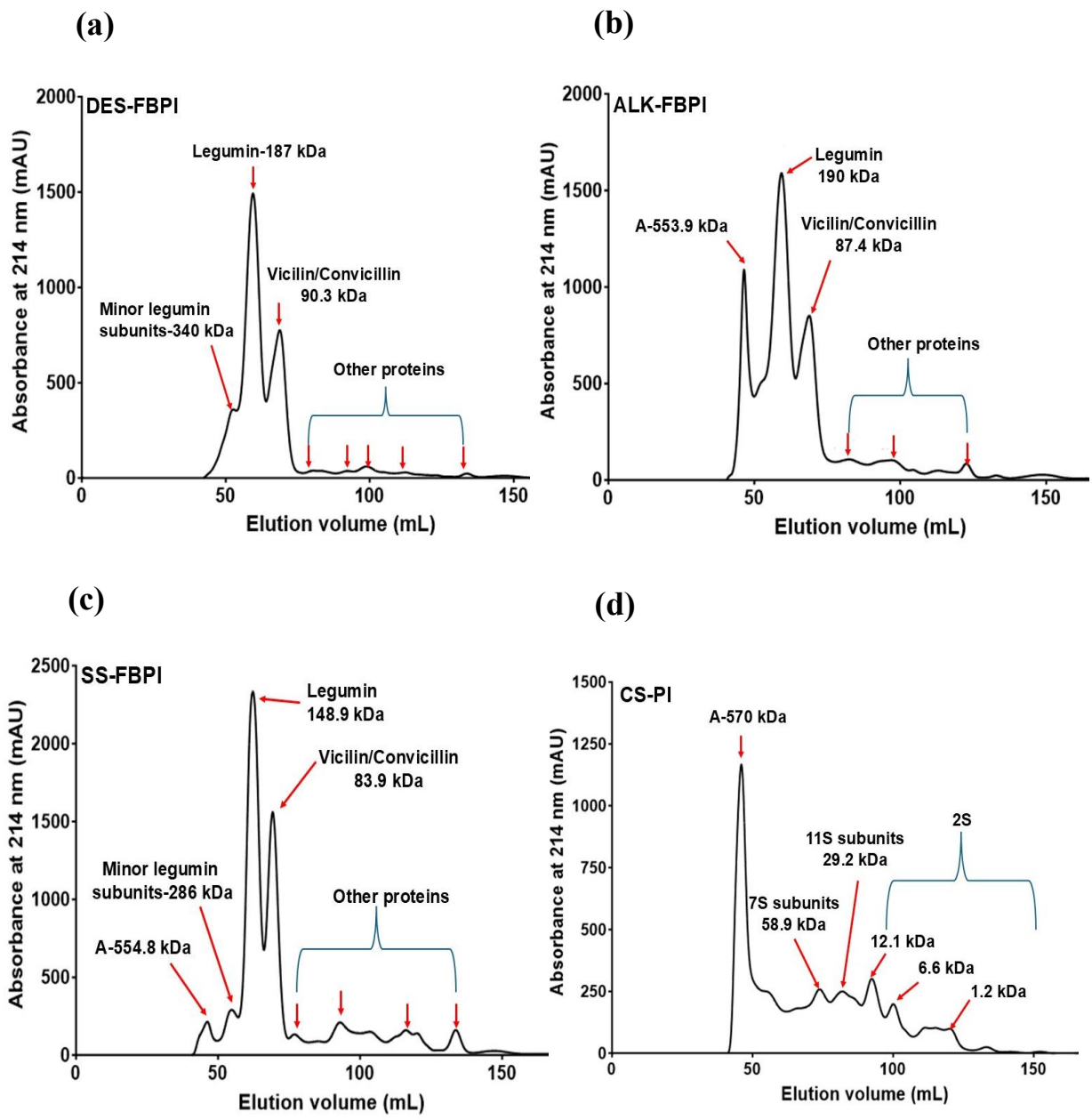


Figure 7. 2. Size exclusion chromatography (SEC) profile (at 214 nm) of proteins extracted by various extraction methods as a function of elution volume (mL), *i.e.* (a)- Deep eutectic solvent-extracted fava bean protein isolates (DES-FBPI), (b)- Alkaline-extracted fava bean protein isolates (ALK-FBPI), (c)- Salt-extracted fava bean protein isolates (SS-FBPI), and (d)- Commercial soy protein isolates (CS-PI).

However, these protein fractions in the SDS-PAGE profile showed as distinct subunits. Several peaks at MWs between 23-45 KDa were detected, assigned to acidic and basic intermediary subunits of legumin as well as subunits of vicilin and convicilin (Mession et al., 2013; Yang et al., 2018). Thus, vicilin/convicilin contains a mixture of subunits with varying proportions. The proteins eluted after the protein standard ribonuclease (13.7 kDa) can correspond to a mixture of albumin fractions, low-molecular-weight vicilin subunits, and heterogeneous polypeptides (Mession et al., 2012; Yang et al., 2018). In Figure 7.2, these protein fractions are collectively referred to as "other proteins" to facilitate easy identification in relation to the major peaks. The areas of the peaks calculated from each spectrum of each protein fraction are shown in Table 7.1. Since one of the significant factors in measuring protein quality is the amount of sulfur-containing amino acids (S-AA), quantifying the relative proportions of major protein classes is essential (Warsame et al., 2020). Among the FBPIs, the legumin fraction of DES-FBPI accounted for the highest peak area (48.36%), followed by SS-FBPI (32.62%) and ALK-FBPI (30.08%), indicating that the DES method is effective in producing high-quality proteins. The 7S fraction of FBPI, on the other hand, had a higher proportion extracted by AE (20.36%) compared to other methods. As evident from the SDS-PAGE electrophoresis profile (Figure 7.1), SE resulted in the disintegration of polypeptide bands attributed to convicilin, as clearly shown by the quantified lowest peak area (14.02%) for SS-FBPI. Generally, the legumin to vicilin/convicilin ratio for fava bean seeds ranges from 2.1 to 3.6, depending on the genotype (Gatehouse et al., 1980), is highly sensitive, and can be affected by different extraction techniques (Warsame et al., 2020). In this study, the legumin to vicilin/convicilin ratio of DES-FBPI and SS-FBPI was ~ 2.71 and ~2.32, respectively, and was comparable to the reported ratio for fava bean seed proteins (Martinez et al., 2016; Shi & Nickerson, 2022). However, ALK-FBPI had a slightly lower legumin to vicilin/convicilin ratio

(~1.48), suggesting that AE tended to extract a slightly higher content of the 7S fraction, while SE and DES extraction were more favorable for legumin (11S) extraction. Similar observations were also reported by Johansson et al.(2023) for AE. Therefore, the isolation technique could strongly impact the functionality of proteins, depending on the extracted legumin to vicilin/convicilin ratio. Furthermore, conventional protein extraction techniques often result in the loss of protein functionality due to denaturation caused by the harsh conditions employed. As expected, CS-PI comprised ~41.11% of the peak area, attributed to high-molecular-weight soluble protein aggregates (A) (Zhang et al., 2024), which is significantly higher than the rest of the extracted FBPIs. Interestingly, the lowest peak area (~8.83%) for the high-molecular-weight soluble protein aggregates (A), along with the small fraction (<2%) of supramolecular aggregates (15S, >15S) (Yang et al., 2018), was observed with DES-FBPI. In the case of ALK-FBPI, soluble aggregates (A) increased almost twice as much as those of DES-FBPI, whereas SS-FBPI had nearly the same amount of soluble protein aggregates as DES-FBPI. Hence, Fava bean proteins extracted using different methods and their evaluated protein compositions will allow the industry to develop high-quality protein ingredients that target desirable functional properties.

7.4.2.2. Changes in Protein Secondary Structures

The changes in protein secondary structure of different protein isolates are presented in Figure 7.1S (Appendix 22) and Table 7.2, respectively. The second derivation of the Amide I region was used to estimate the secondary structure components, as it is most sensitive to C=O stretching vibrations (80%) of the peptide bond, followed by C-N and N-H bending (Carbonaro & Nucara, 2010). The peaks assigned to structural components were β sheets, 1618-1640 cm^{-1} and 1680-1688 cm^{-1}, α-helix, 1650-1660 cm^{-1}, random coil, 1640-1650 cm^{-1}, β-turn, 1660–1700 cm^{-1} and 1670-1680 cm^{-1}, and protein aggregates (intermolecular β-sheets structure / amino acid side

chains), 1690-1695 cm^{-1} and 1610-1615 cm^{-1}, respectively (Shevkani et al., 2019). For all protein fractions isolated, the major secondary structure component was β-sheets. These results agreed with those reported for protein extracted by isoelectric precipitation from fava beans (Nivala et al., 2021). No significant difference ($P > 0.05$) was found among the samples in β-sheet contents. However, the amounts of α-helices, β-turns, and protein aggregates present in DES-FBPI was significantly lower ($P < 0.05$) than in all other isolates. ALK-FBPI and SS-FBPI, on the other hand, did not exhibit significant differences in their structural components. In addition, SC-PI showed a significantly higher percentage of α-helices, β-turns, and protein aggregates compared to the other isolation techniques, plausibly due to the industrial protein isolation method. The differences in the structural components induced by the different extraction methods indicate the potential influence on the physiochemical characteristics of proteins, thus relating to techno-functional properties.

Table 7. 2. The relative proportions of secondary structure components of extracted protein isolates

Components	β-sheets %	α-helix %	β-turns %	Unordered structures %	Aggregates%
DES-FBPI	29.86±2.56^{a}	9.88±0.38^{b}	8.04±0.39^{b}	4.37±0.11^{a}	5.30±0.11^{b}
ALK-FBPI	23.30±1.42^{a}	11.04±0.64^{ab}	11.07±1.47^{ab}	4.03±0.35^{a}	5.50±0.25^{ab}
SS-FBPI	25.82±4.86^{a}	10.47±0.27^{ab}	10.56±0.34^{ab}	4.12±0.45^{a}	5.44±0.07^{ab}
CS-PI	26.23±2.29^{a}	11.37±0.41^{a}	14.81±4.23^{a}	7.20±3.15^{a}	6.20±0.54^{a}

Relative proportion (%) of secondary structure components analyzed from the Amide I peak with FITR analysis after the protein isolate obtained by different extraction methods *i.e.* (a)- Deep eutectic solvent-extracted fava bean protein isolates (DES-FBPI), (b)- Alkaline-extracted fava

bean protein isolates (ALK-FBPI), (c)- Salt-extracted fava bean protein isolates (SS-FBPI), and (d)- Commercial soy protein isolates (CS-PI). All data are presented as means ± standard deviation and were analyzed using analysis of variance (ANOVA) followed by Tukey's test (n=3). Different letters in the column indicate a significant difference ($P < 0.05$).

7.4.2.3. Changes in Intrinsic fluorescence (IF)

The intrinsic fluorescence of proteins provides information about the protein's tertiary structural changes, such as protein folding and unfolding. The aromatic amino acids tryptophan, tyrosine, and phenylalanine can absorb energy and emit fluorescence light, which can be used to evaluate the degree of amino acid exposure, thereby indicating a change in protein conformation (Keeratiurai et al., 2012). Particularly, the intrinsic fluorescence properties of tryptophan are sensitive to solvent polarity (Ghisaidoobe & Chung, 2014). When tryptophan residues are buried inside the protein's core, i.e., in the folded state (hydrophobic environment), intensity becomes high, whereas, in the partial or complete folded state of proteins (hydrophilic environment/exposure to the solvent), it has a reduced fluorescence intensity (Rahman & Lamsal, 2023). Therefore, the tryptophan spectrum can be used to identify conformational changes in proteins. As shown in Figure 7.3 (a), DES-FBPI and SS-FBPI showed higher fluorescence intensities than ALK-FBPI. However, the fluorescence intensity of CS-PI decreased remarkably compared to all other protein isolates. In addition, the maximum emission wavelengths of CS-PI and ALK-FBPI, DES-FBPI, and SS-FBPI were 336 nm (redshift) and 316-318 nm (blueshift), respectively. This indicates that DES-FBPI and SS-FBPI have lower denaturation (more compact state) than the alkaline process (partially denatured), but the most extensive conformational changes occurred for CS-PI. Thus, aromatic amino acid residues in CS-PI were exposed to a more hydrophilic environment, which was accompanied by the harsh conditions used in industrial

processing. In contrast to our study, Yang et al. (2021) found that alkaline-extracted pea proteins induced higher fluorescence intensities compared to those obtained through salt extraction. Similar observations were also reported by Jiang et al. (2009) for soy proteins. The differences in extraction conditions and raw materials may have contributed to the varied outcomes of our study.

7.4.2.4. Free SH content

The methionine and cysteine content in fava beans ranges from 2.6 to 3.7 g/kg of dry matter, respectively (Vioque et al., 2012). Sulfhydryl groups (-SH) play a vital role in protein functionality, as inter- and intra-molecular disulfide bonds (-S-S-) form. –SH groups can be transformed into each other under protein processing and modifications (Shen et al., 2022). However, the relative content of free-SH content may vary in isolated proteins under different extraction conditions, resulting in variations in protein functionality. Figure 7.3 (b) shows the free sulfhydryl content in each protein fraction. A significantly higher ($P < 0.05$) free–SH concentration was found with CS-PI, followed by ALK-FBPI, SS-FBPI, and DES-FBPI. Commercial processing of protein isolates under high-heat treatment can result in exposure to buried –SH groups. This may lead to higher accessibility of free SH groups on protein surfaces. Therefore, the degree of protein folding determines the available free–SH groups. Based on the intrinsic fluorescence results [Figure 7.3(a)], it was observed that the degree of denaturation was higher for ALK-FBPIs compared to SS-FBPI, likely due to the high pH involved in the extraction. Hou et al. (2017) reported that the alkaline concentration (> 0.03 M) used in the extraction of rice protein isolates significantly decreased the disulfide and free-SH content than that of the equal or lower alkaline concentration of 0.03 M. Overall, a significant reduction of free –SH groups in DES-FBPI suggests that DES extraction induces a lower degree of denaturation through its mild extraction process, thereby decreasing the availability of –SH groups.

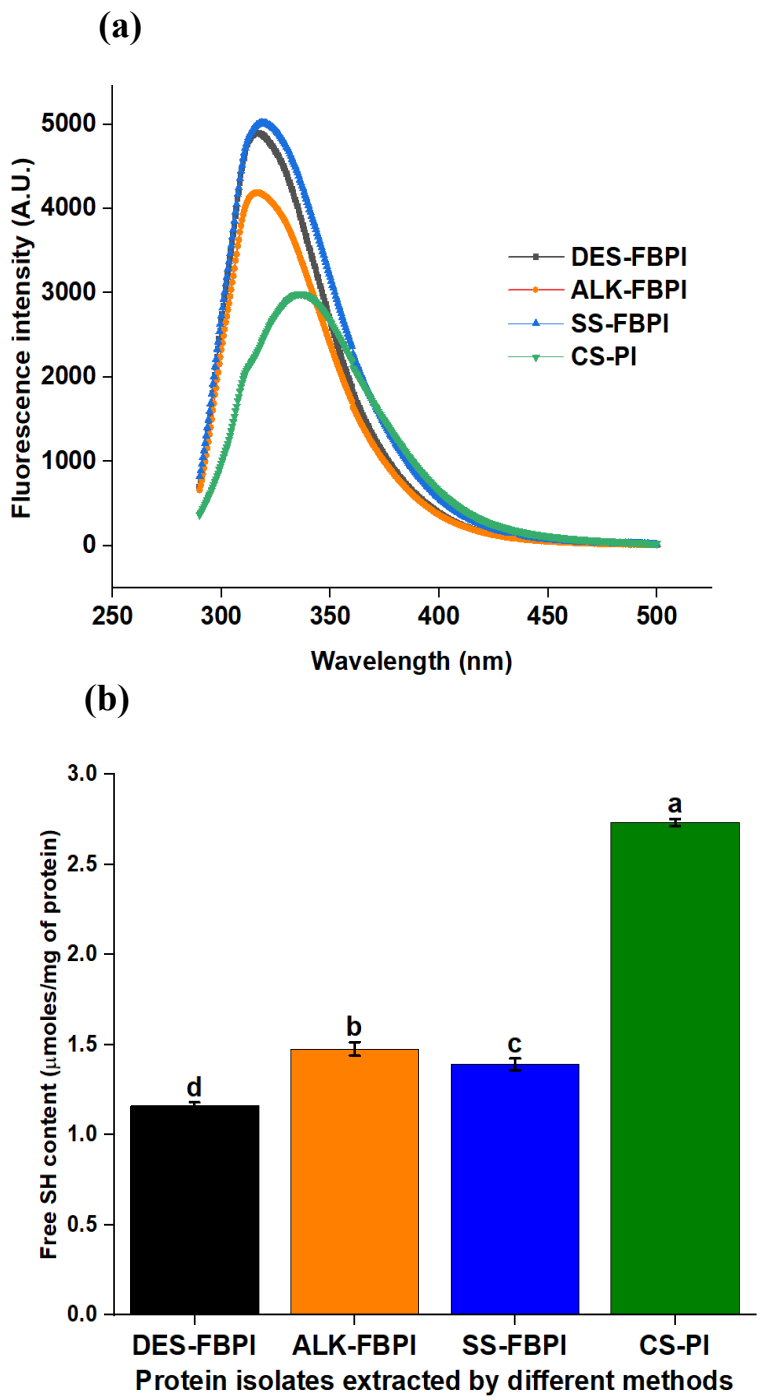


Figure 7. 3 (a). Intrinsic fluorescence, **(b)-** free sulfhydryl content (µmoles/mg of proteins) of proteins extracted by various methods *i.e.* Deep eutectic solvent-extracted fava bean protein isolates (DES-FBPI), Alkaline-extracted fava bean protein isolates (ALK-FBPI), Salt-extracted fava bean protein isolates (SS-FBPI), and Commercial soy protein isolates (CS-PI). All data are

presented as means ± standard deviation, including error bars, and were analyzed by analysis of variance (ANOVA) followed by Tukey's test (n=3). Different letters at the bars indicate a significant difference ($p < 0.05$).

7.4.2.5. Changes in Protein Surface Functional Groups

X-ray photoelectron spectroscopy (XPS) can be used to determine the elemental composition of the protein's surface layer, the oxidation state of the elements, and surface functional groups (Dombrowski et al., 1995). The elemental composition of all protein isolates obtained from the different extractions is shown in Table 7.4S (Appendix 26). There were three predominant peaks in the survey spectra related to the main elements of the protein backbone: carbon (C1s, ~ 284.5 eV), Nitrogen (N1s, ~ 398 eV), and oxygen (O1s, ~ 530 eV). A very small peak for sulfur (S 2p) was observed in each spectrum, but its percentage was below 1% for all isolates, except for ALK-FBPI, which had a value of 0%. The results showed that the surface properties of the DES-FBPI differed from those of other isolates obtained using the SE and AE methods (Table 7.4S- Appendix 26). A relatively high C composition was observed in all protein isolates, with greater similarity between DES-FBPI (78.81%) and CS-PI (78.88%) compared to SS-FBPI (75.80%) and ALK-FBPI (75.45%). However, the N percentage of DES-FBPI (7.21%) was slightly lower compared to that of ALK-PBPI (8.71%) and SS-FBPI (8.65%) but was higher than that of CS-PI (5.55%). Furthermore, the O atom composition in DES-FBPI also slightly decreased (13.73%) compared to all other isolates (15.25-15.85%). These results indicated that the composition of DES-FBPI's surface differed from that of its fava bean counterparts. Different factors can contribute to these compositional differences in the protein surface. The major driving force behind the pure DES structure is believed to be hydrogen bonding (HB) (Liu et al., 2018). It has been shown that adding water to DES could disrupt the HB network (Gabriele et al., 2019).

Since DES extraction was performed with a mixture of 40% (w/w) water and 60% DES (w/w) in this study, the HB interaction between protein and DES may have weakened, leading to a lower interaction with charged groups on the surface. This might change the band frequency of the C, N, and O atoms on the surface of proteins. On the other hand, DES-water interaction can alter the surface structure of proteins (Yue et al., 2021), thereby affecting the band frequency of the XPS spectra. Moreover, the ratio of the peak areas of C 1s, N 1s, and O 1s presented in Table 7.4S (Appendix 26) provides the changes in the concentration of these atoms on each protein surface. As observed, the decreased N/C ratio of DES-FBPI compared to ALK-FBPI and SS-FBPI suggests that the protein surface may have slightly fewer amino acids exposed, possibly due to a slight Millard reaction promoted by the heat-induced DES extraction (Nasrollahzadeh et al., 2023). This was more prominent with the CS-PI as it had the lowest N/C ratio among the protein isolates, consistent with Li et al. (2015). Interestingly, the O/C atom ratio of DES-FBPI was lower than that of all other isolates, suggesting that DES extraction may lead to less protein oxidation compared to conventional methods. This suggests that the oxidation state of elements leads to a change in binding energy, providing new molecular interactions formed upon DES extraction.

The high-resolution spectra of C1s, N1s, and O1s can be used to identify surface functional groups in various protein isolates (Zhao et al., 2015). The binding energies and areas corresponding to each functional group, analyzed for all proteins extracted from various methods, are presented in Figures 7.4A, B, and C, as well as in Table 7.5S (Appendix 27). High-resolution C1s spectra revealed three main functional groups in all protein isolates: C-C/C-H, H-C-H, and C=O (Guerrero et al., 2013; Zhao et al., 2015). According to Figure 7.4A, the spectral shape and the band position of C1 for all protein isolates seemed similar. This implied a similar distribution of C-C and C-H on the protein surface, obtained from the different isolating techniques. However, the spectral

shape and the band position of H-C-H and C=O differed among the isolates. Considering the binding energy and the content of each functional group (Table 7.5S in Appendix 27), the major peaks, C-C/C-H and H-C-H, were identified at 284.40-284.60 eV and 285.50-285.85 eV, respectively, corresponding to the hydrocarbon and C-C bonding. The C=O band, found at a binding energy range of 287.66-287.68 eV, represented the ketone and aldehyde groups, consistent with the literature (Zhao et al., 2015). The variation in the binding area of H-C-H and C=O reflects the change in the C element content induced by the different extraction methods. The functional groups of C-C/C-H and H-C-H indicate the hydrophobic characteristics, while C=O dictates the hydrophilic properties. The binding area with C-C/C-H for CS-PI was higher, followed by DES-FBPI, ALK-FBPI, and SS-FBPI. However, binding areas for C=O were found to be higher in DES-FBPI than in other proteins. At the same time, hydrocarbon (H-C-H) with the C=O band of DES-FBPI had the lowest binding area compared to its other counterparts. These results indicated that ALK-FBPI and CS-PI surfaces were more hydrophobic than other protein isolates. In contrast, SS-FBPI and DES-FBPI appeared to have more hydrophilic bonding surfaces, which was consistent with the intrinsic fluorescence results shown in Figure 7.3(a). Despite these findings, it is worth noting that DES extraction had a lower O/C ratio (0.17) compared to the other isolates (0.20-0.21) (Table 7.4S in Appendix 26). This suggested that protein obtained from the DES methods could be less oxygenated than that from conventional methods.

For the N1s spectra (Figure 7.4B), three peaks were observed for all protein samples and were assigned as N-H/C-N at bonds at 398.89-398.99 eV, C=N/C-N at 399.01-399.6 eV, and amide N at 400.10 eV (Guerrero et al., 2013). The shape and positions of the N1s spectra bands for ALK-FBPI and CS-PI were similar, but the rest of the two protein samples, DES-FBPI and SS-FBPI, differed. However, the band intensities for the N1s spectra varied among all samples. The reason

could be the different proportions of exposed amino groups towards the protein surface (Clark et al., 1976). The binding area for the N-H/C-N bond from ALK-FBPI was higher than that of the protein obtained using other techniques; however, the protein from DES extraction did not exhibit a significantly larger difference. Moreover, the amide N band binding energy area was greater in DES-FBPI, indicating that DES extraction may affect the N1s binding energy. The lower binding energy of CS-PI and SS-FBPI may be attributed to the adsorption onto decomposed protein powders or the higher content of neutral amino groups (N-H) on the surface (Clark et al., 1976).

Figure 7.4C shows the O1s components of the surfaces of all protein isolates. The O1s were decomposed into three peaks for DES-FBPI and ALK-FBPI, and two for the remaining protein isolates. The former corresponded to the O-C=O, O-C=O/O=C-N, and C-O-C/C-OH functional groups, while the latter corresponded to the O-C=O and C-O-C/C-OH peaks in SS-FBPI and CS-PI (Zander et al., 2012). The missing oxygen functional group of the C-O-C/C-OH peak in SS-FBPI and CS-PI reflects that salt extraction and commercial production of soy proteins were more prone to oxidation, agreeing with the results obtained for the O/C ratio in Table 7.4S (Appendix 26) and the two broader peaks observed in Figure 7.4C. The O1s featured a similar shape for all proteins, but the peak positions differed. Different extraction methods have induced different oxygen-containing surface functional groups in the proteins. The major component identified in O1s was the O-C=O, followed by the O-C=O/O=C-N and C-O-C/C-OH, among which the binding area of O-C=O/O=C-N in DES-FBPI was found to be lowest. Hence, these results suggest that DES protein extraction results in less protein oxidation compared to conventional methods.

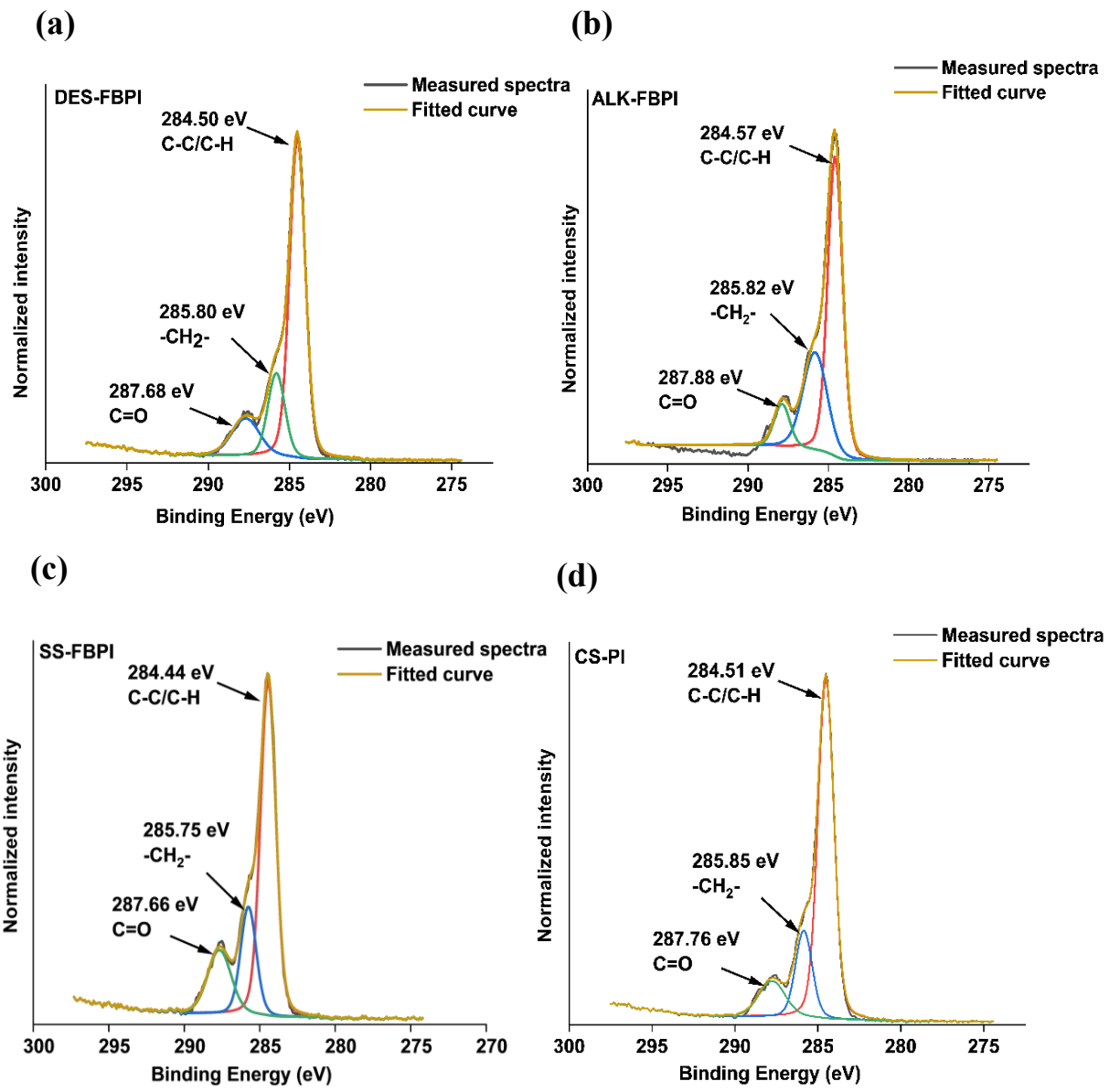


Figure 7. 4A. Fitted peaks of XPS spectra for C1s in fava bean protein isolates extracted from various extraction methods; *i.e.* (a)- Deep eutectic solvent-extracted fava bean protein isolates (DES-FBPI), (b)- Alkaline-extracted fava bean protein isolates (ALK-FBPI), (c)- Salt-extracted fava bean protein isolates (SS-FBPI), and (d)- Commercial soy protein isolates (CS-PI).

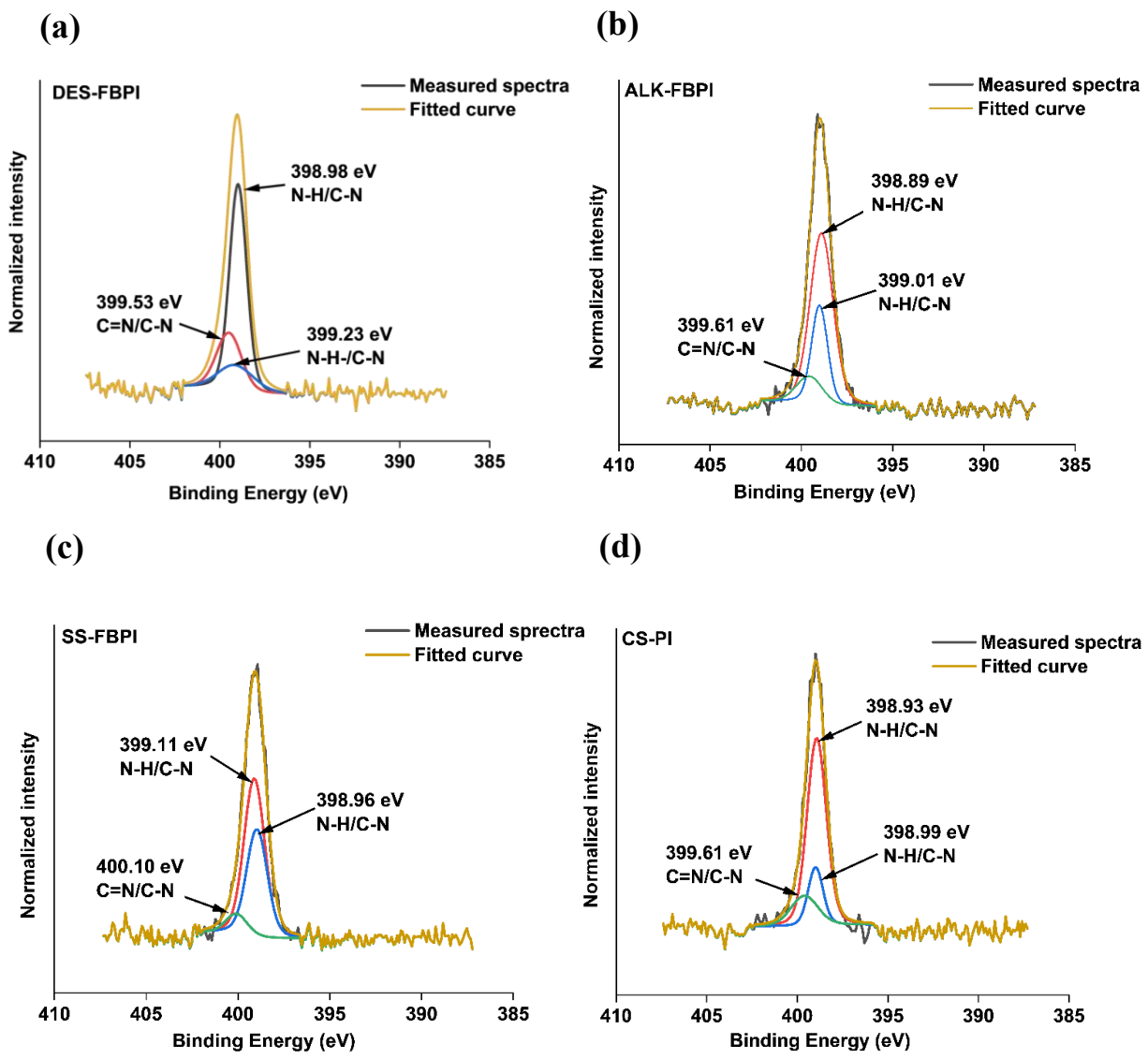


Figure 7. 4B. Fitted peaks of XPS spectra for N1s in fava bean protein isolates extracted from various extraction methods; *i.e.* (a)- Deep eutectic solvent-extracted fava bean protein isolates (DES-FBPI), (b)- Alkaline-extracted fava bean protein isolates (ALK-FBPI), (c)- Salt-extracted fava bean protein isolates (SS-FBPI), and (d)- Commercial soy protein isolates (CS-PI).

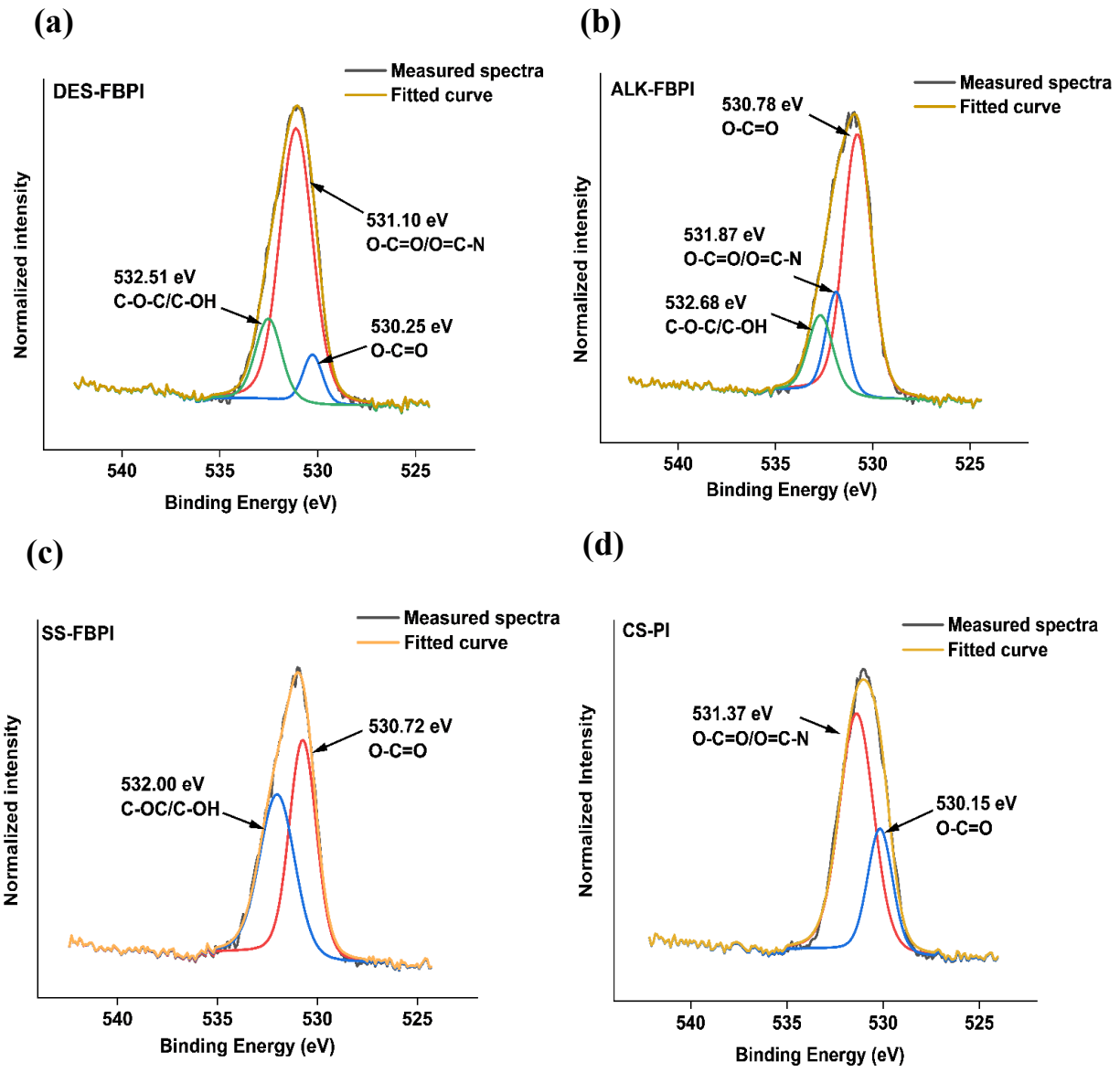


Figure 7. 4C. Fitted peaks of XPS spectra for O1s in fava bean protein isolates extracted from various extraction methods; *i.e.* (a)- Deep eutectic solvent-extracted fava bean protein isolates (DES-FBPI), (b)- Alkaline-extracted fava bean protein isolates (ALK-FBPI), (c)- Salt-extracted fava bean protein isolates (SS-FBPI), and (d)- Commercial soy protein isolates (CS-PI).

7.4.3. Effect of protein extraction methods on fava bean protein gelation

7.4.3.1. Least gel concentration (LGC) of protein isolates

The LGC was measured at protein concentrations ranging from 5% to 20% (w/v) and pH levels from 3.0 to 9.0 for all protein isolates. As shown in Figure 7.5, DES-FBPI formed a firm, stand-alone gel at a 20% (w/v) protein concentration (pH 7.0), whereas ALK-FBPI and SS-FBPI, under the same conditions, formed self-standing gels with uneven, soft texture and weak handling properties. In the case of CS-PI, self-standing gels formed between 12% and 20% (w/v) protein concentration (pH 7.0), indicating superior gel formation at a lower protein concentration of 12%. [Table 7.1S (Appendix 23)]. Therefore, for a fair comparison, gels from each isolate were prepared at 20% (w/v) protein concentration and pH 7.0 to evaluate subsequent gelation properties. Herein, gels prepared from DES-FBPI, ALK-FBPI, SS-FBPI, and CS-PI, referred to as DES-extracted fava bean gels (DES-FBG), ALK-extracted fava bean gels (ALK-FBG), SS-extracted fava bean gels (SS-FBG), and Commercial soy protein gels (CSG), respectively.

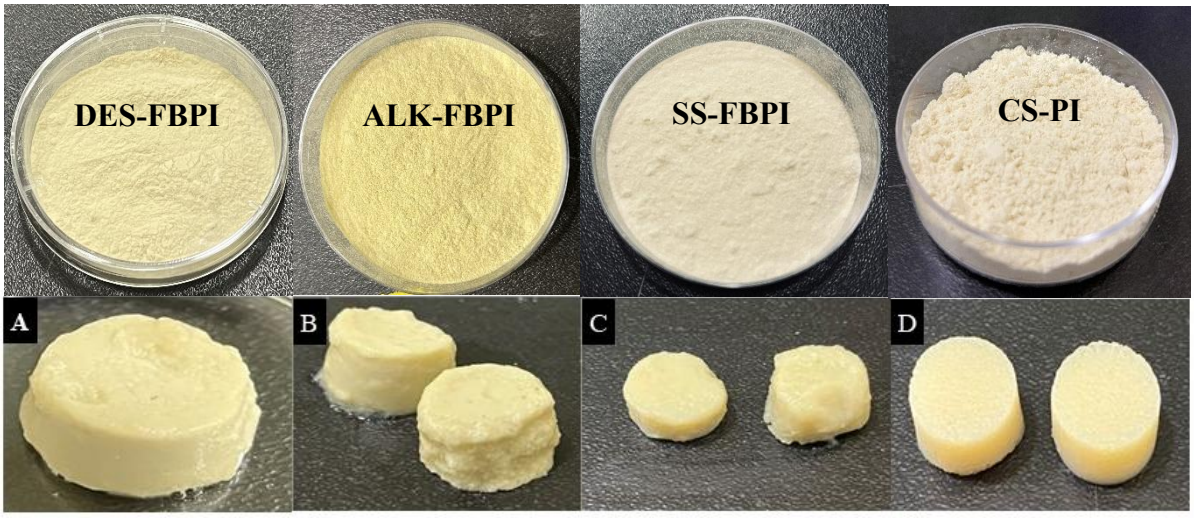


Figure 7. 5. Visual appearance of protein isolates and corresponding gels formed (20% w/v protein concentration and pH 7.0) by various extraction methods *i.e.* **(A)-** Deep eutectic solvent-extracted

fava bean gels (DES-FBG), **(B)-** Alkaline-extracted fava bean gels (ALK-FBG), and **(C)-**Salt-extracted fava bean gels (SS-FBG), and **(D)**- Commercial soy protein gels (CSG).

7.4.3.2. Effect of protein extraction methods on the changes in rheological properties

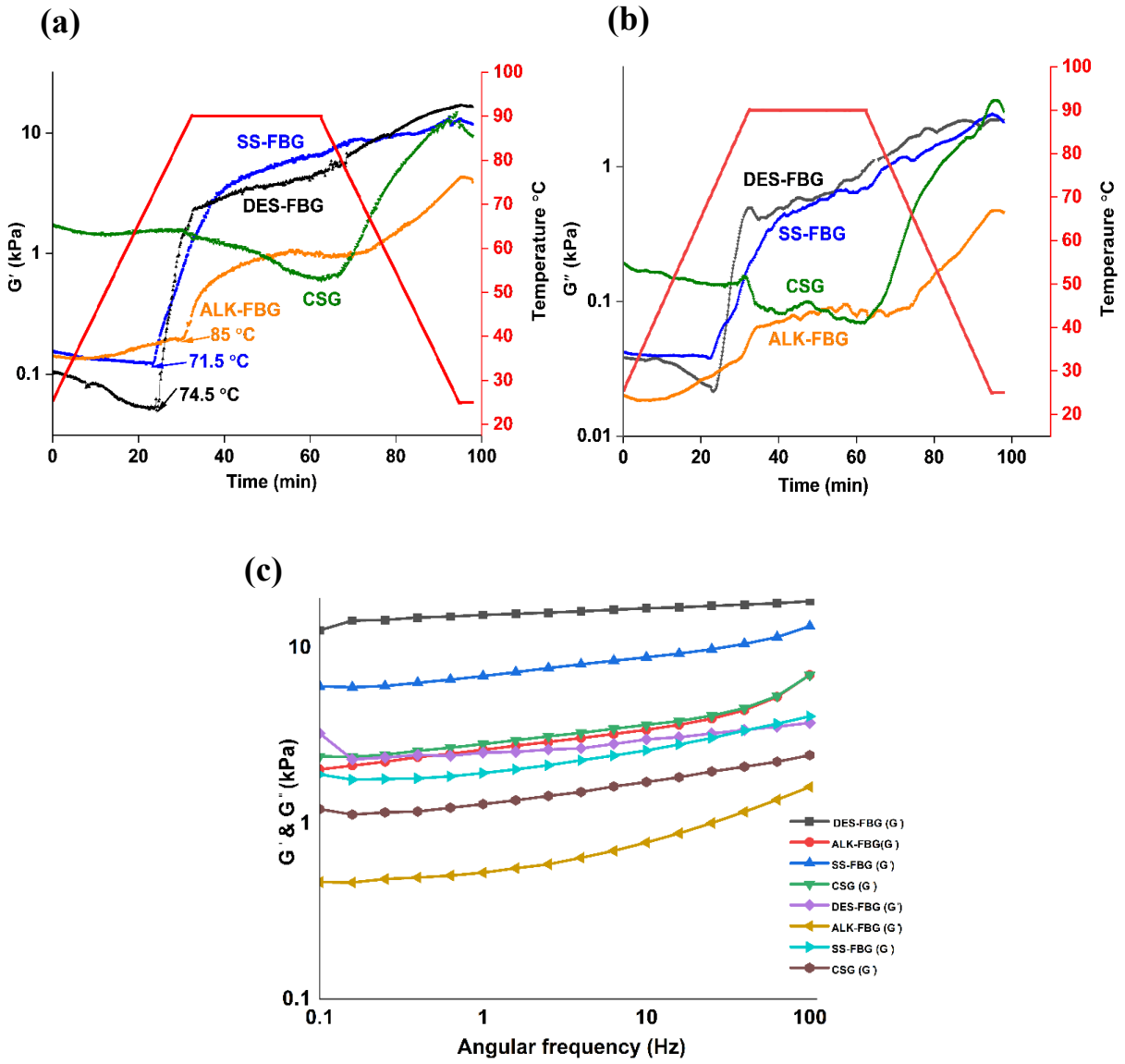


Figure 7. 6. Rheological characterization of gels prepared (pH-7.0, protein concentration 20% w/v) from protein isolates obtained from various extraction methods *i.e.* Deep eutectic solvent-extracted fava bean gels (DES-FBG), Alkaline-extracted fava bean gels (ALK-FBG), Salt-

extracted fava bean gels (SS-FBG), and Commercial soy protein gels (CSG), and graphs represents the effect of the heat-induced gelation process on (a) storage modulus (G'), (b) loss modulus (G"), (c) frequency dependence on storage modulus (G'), and loss modulus (G"). All data are presented as means ± standard deviation and were analyzed by analysis of variance (ANOVA) followed by Tukey's test (n=2), $P < 0.05$.

A small deformation rheology test was conducted to investigate the gelation behavior of protein dispersions prepared from fava bean protein isolates extracted using various methods. In rheology, the storage modulus (G') represents the elasticity components, while the loss modulus (G") accounts for the viscous component of a material (Batista et al., 2005; Zha et al., 2021). Small deformation rheology provides important information about the development of gel properties during the entire gelation process. Figure 7.6 and Table 7.3 show the rheological behavior and changes in gelation properties of each protein isolate during the heating and cooling cycle, respectively. During heating, G' was initially low for all protein suspensions made from fava bean protein isolates, and then G' was further decreased gradually up to a critical temperature, after which G' increased rapidly. This indicated that a certain amount of protein needs to be denatured before the onset of gel formation, as gel transitions occur from a protein suspension to a solid-like structure. The temperature at which G' rapidly increased is defined as the gel initiation temperature (T_g). Usually, during heat-induced gelation, the transition of a viscous material into a viscoelastic gel (gel point) occurs when the G' < G" to G' > G" (Johansson et al., 2023), referring to the gel transition point. In our study, however, all cases showed G' > G" from the beginning of the temperature sweep. Thus, there was no gel transition point but only the T_g.

The T_g value of DES-FBPI was approximately 74.5 °C, slightly higher than that of SS-FBPI (~71.5 °C) and considerably lower than that of ALK-FBPI (85 °C), which fell within the

reported range for fava bean proteins (Nilsson et al., 2022). Typically, globular proteins exhibit higher gelation temperatures than their denaturation temperatures, as the unfolding of proteins exposes their interior hydrophobic sites, which form a three-dimensional gel network through covalent and non-covalent molecular interactions required for gelation (Yang et al., 2021). The higher T_g value of ALK-FBPI reflected resistance to thermal denaturation, which was likely due to conformational changes in the protein structure. Also, the higher gelation temperature of ALK-FBPI may be related to its slower gelation kinetics caused by residual NaCl from acid precipitation. On the other hand, the gelling behavior of CS-PI was different, as both G' and G" were initially higher than those of all other protein dispersions and progressively decreased as the temperature was raised. Initially, it was unclear why both moduli decreased despite their higher G' and G". Theoretically, prolonged heating should increase the moduli as more protein is incorporated into the network and subsequent structural rearrangement within the protein matrix. Hence, the possible reason for the initial decreased moduli of CS-PI can be attributed to the breakdown of aggregates. Moreover, the dissociation of globulin subunits, due to the disruption of hydrogen bonds and increased entropy during the heating cycle, may be responsible for the initial decreased moduli of CS-PI (Chronakis et al., 1995; Comfort & Howell, 2002). Additionally, it was observed that the CS-PI suspension produced a viscous (gel-like) and thick paste at a 20% (w/v) protein concentration (pH 7.0), even at the solubilization temperature of 23 °C (Temperature at which protein suspension was prepared). This was even clearly evident with the significantly higher viscosity values of CS-PI suspension (Table 7.3), compared to all other counterparts. This may be why the initial G' and G" values were high in CS-PI. Consequently, protein denaturation and aggregation may be avoided even when heated at 90 ºC due to decreased aggregate interactions (Chronakis et al., 1995). Therefore, the T_g value of CS-PI could not be determined. In the next

stage of CS-PI, the cooling cycle progressively increased G' and G" (G' > G"), which could be attributed to the development of non-covalent interactions among aggregates due to the diminished entropy in the system (Chronakis et al., 1995; Chronakis & Kasapis, 1993).

In the temperature sweep studied for different FBI dispersions, the G' values increased (G' > G") rapidly after the T_g until 90 °C. It was observed that the increase of G' for DES-FBPI and SS-FBPI was higher than that of ALK-FBPI, indicating higher protein unfolding and presumably strong intermolecular interactions between protein aggregates of DES-FBPI and SS-FBPI. The higher degree of protein denaturation achieved through alkaline extraction may hinder further unfolding and aggregation during heating, thereby reducing intermolecular interactions. This was consistent with the results obtained for the intrinsic fluorescence [Figure 7.3(a)]. During the temperature holding up at 90 °C for 30 min, the G' value of SS-FBPI surpassed that of DES-FBPI, indicating a higher protein aggregation rate. However, when the temperature cooled down, the G' value of DES-FBPI again increased, exceeding that of SS-FBPI until the end of the cooling cycle. Hence, the final G' values of all gels significantly differed ($P < 0.05$); DES-FBG was the strongest (16.39 ± 0.30 kPa), followed by SS-FBG (11.85 ± 2.65 kPa), CSG (9.41 ± 0.13 kPa), and ALK-FBG (3.91 ± 0.36 kPa). It can be deduced that all gels were initially stabilized predominantly by hydrophobic interactions, due to the increase of G' during the heating stage, resulting from continuous protein incorporation into the network, and to a lesser extent, covalent bonds (Janssen et al., 2024). Therefore, the increased moduli during cooling were possibly due to covalent crosslinks (disulfide interactions), electrostatic interactions, and hydrogen bond interactions. (Tanger et al., 2022). However, no distinction could be made in the final loss tangent, tan(δ) value (G"/G'), or final G" as they were found to be the same ($P > 0.05$) among all gels, including CSG. Notably, the δ value remained below one for all samples. If a material is in a viscous liquid state,

tan (δ) is equal to one or below 1, whereas samples with tan (δ) < 1 exhibit gel characteristics, with lower values (< 0.1) associated with high rigidity, strong gels (Pereira et al., 2022). Interestingly, the tan (δ) value for FBG samples sharply declined after the $T_{g,}$ with only ALK-FBPI and SS-FBPI continuing to decrease further until the end of the heating cycle. Then, it increased again during the cooling stage (Figure 7.2S- Appendix 28). For DES-FBG, in particular, the tan (δ) was decreased initially during the heating stage, similar to other FBGs. However, during the temperature holding at 90 °C for 30 min, the tan (δ) value of DES-FBG was increased compared to SS-FBG and the ALK-FBG, and then it levelled off at the beginning of the cooling cycle until halfway through the cooling stage. After that, the tan (δ) value decreased sharply, reaching a value of 0.13 at the end of the cooling stage. The levelling off of the tan (δ) value of DES-FBG during the cooling stage means that both G' and G" do not change, thus gel formation becomes homogenous, leading to a strong gel network.

The frequency sweep spectra obtained for the gel samples, based on frequency dependence (1% strain), are shown in Figure 7.6(c). The frequency dependence of G' provides information about the mechanical properties of gels (Zhao et al., 2021). G' was greater than G" for all samples within the studied frequency range, and each modulus increased slightly as the angular frequency increased, indicating the viscoelastic characteristics of the gels. The DES-FBG showed a significantly higher G' (18.30 ± 0.50 kPa), followed by SS-FBG (13.27 ± 4.52 kPa), and ALK-FBG (7.00 ± 1.07 kPa) and CSG (7.00 ± 2.82 kPa). The frequency dependence can be analyzed by calculating the slope (n) and intercept (K) of the curve of G′ as a function of frequency (ω) from the equation, log (G′) = n*log(ω) + K (Tanger et al., 2022). When the slope is closer to zero, the gel matrix is said to mainly be formed mainly by covalent bonds. In contrast, slightly higher frequency dependence is associated with hydrogen bonding and hydrophobic interactions (Tanger

et al., 2022). The data shows that both G' and G" for DES-FBG depended relatively little on the frequency sweep applied compared to all other gels. This implies that the network formed might have relatively permanent intramolecular and intermolecular rearrangements of protein molecules with a long relaxation time.

Table 7. 3. Gelation properties of extracted protein isolates

Protein gels	T_g (°C)	Gelling Properties						
		Heating (90 °C) /Cooling (25 °C)		Tan (δ)	Frequency sweep		Viscosity (Pa. s)	
		Final G'(kPa)	Final G'' (kPa)	Final G''/G'	Final G'(kPa)	Final G'' (kPa)	1 /s	500/ s
DES-FBG	74.5	16.39±0.30^{a}	2.21±0.11^{a}	0.13±0.01^{a}	18.30±0.50^{a}	3.73±0.46^{ab}	0.017±0.002^{b}	0.005±0.00^{b}
ALK-FBG	85	3.91±0.36^{c}	0.88±0.17^{a}	0.22±0.02^{a}	7.00±1.07^{b}	1.61±0.34^{b}	0.041±0.02^{b}	0.010±0.00^{b}
SS-FBG	71.5	11.85±2.65^{ab}	3.05±1.42^{a}	0.25±0.06^{a}	13.27±4.52^{ab}	4.07±0.79^{a}	0.030±0.001^{b}	0.005±0.00^{b}
CSG	ND	9.41±0.13^{b}	4.13±1.34^{a}	0.44±0.15^{a}	7.00±2.82^{b}	2.44±0.52^{ab}	242.20±32.20^{a}	0.423±0.14^{a}

Rheological properties [Storage modulus (G'), loss modulus (G")] of gels prepared (pH-7.0, protein concentration 20% w/v) from protein isolates obtained from various extraction methods *i.e.* Deep eutectic solvent-extracted fava bean gels (DES-FBG), Alkaline-extracted fava bean gels (ALK-FBG), Salt-extracted fava bean gels (SS-FBG), and Commercial soy protein gels (CSG). All data presented as means ± standard deviation were analyzed by analysis of variance (ANOVA) followed by Tukey's test (n=3). Different letters in each column indicate a significant difference ($P < 0.05$). ND- Not detected.

7.4.3.3. Intermolecular bonds involved in gel formation and the mechanism

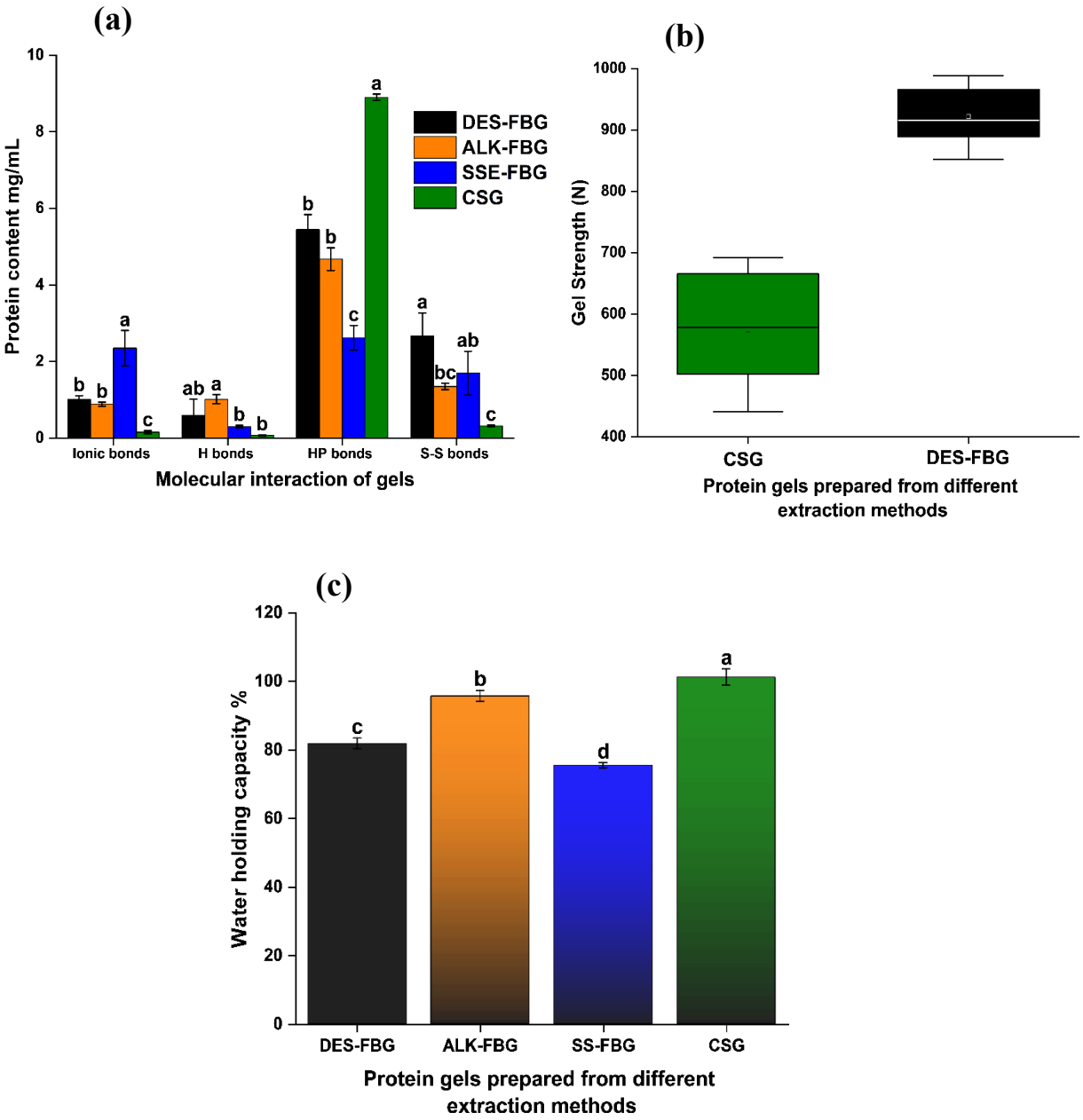


Figure 7. 7. (a)- water holding capacity of gels, **(b)-** gel strength (N) analyzed by texture analyzer, and **(c)** molecular interactions of gels as a function of protein content (mg/mL) of protein isolates extracted by various methods *i.e.* Deep eutectic solvent-extracted fava bean gels (DES-FBG), Alkaline-extracted fava bean gels (ALK-FBG), Salt-extracted fava bean protein gels (SS-FBG),

and Commercial soy protein gels (CSG). All data presented as means ± standard deviations were analyzed by analysis of variance (ANOVA) followed by Tukey's test (n=3). Different letters at the bars indicate a significant difference ($P < 0.05$).

Figure 7.7(a) and Table 7.2S (Appendix 24) illustrate the relative content of intermolecular forces in prepared gels obtained using different extraction methods. Protein gels treated with denaturing solvents can disrupt various interactions. Ionic bonds can be broken with NaCl (0.6 mol/L), while hydrogen bonds can be broken with urea (1.5 mol/L). At higher concentrations (8 mol/L urea), urea can disrupt the hydrogen bonds and hydrophobic interactions, whereas 0.5 mol/L β-mercaptoethanol could disrupt disulfide bonds (Xu et al., 2010). The molecular forces involved in the gel network can be influenced by the method used for protein isolation, in which salts, pH, temperature, and solvent may alter the protein conformation, thereby modulating different interaction forces in the formation of the gel network (Sun & Arntfield, 2012). As shown in Table 7.2S (Appendix 24), hydrogen bonds, ionic bonds, hydrophobic interactions, and disulfide bonds were involved in protein gelation. CSG consisted of significantly higher ($P < 0.05$) hydrophobic interactions than all other cases, which played a primary role in gel formation, consistent with the previously reported data (Fu et al., 2023; Van Kleef, 1986). This was further supported by the higher total hydrophobic amino acid (AA) content in the CS-PI (Table 7.3S – Appendix 25). However, the effect of other molecular forces in the CSG gel matrix was minimal. In the literature, the native soy proteins have exhibited clear transition points in the temperature sweeps of individual globulins (Hua et al., 2005). Our results demonstrated that the gelling process of commercial soy proteins differs from that of their native counterparts.

Figure 7.8(a) illustrates the mechanism of heat-induced gel formation in commercial soy isolates. Commercially produced soy proteins are partially denatured due to the industrial process.

This produces a viscous, cream-colored, solid-like paste at the solubilized temperature (room temperature). When heated, the viscosity of the system decreases, and it gains entropy (energy stored), causing the hydrogen bonds to rupture more quickly and weakening the molecular association. As a result, electrostatic cross-links between protein aggregates are diminished. This whole process can be regarded as "disaggregation." After cooling, the denatured proteins reinforce the formation of the three-dimensional network, primarily through hydrophobic interactions and, to a lesser extent, through other interactions. On the other hand, gels formed by fava bean proteins using different isolation methods appeared to be stabilized by a combination of both covalent and non-covalent bonds, with non-covalent bonds, specifically hydrophobic interactions, being predominant in all gels (Table 7.2S-Appendix 24). It was observed that the latter were significantly higher in both DES-FBPI and ALK-FBPI than in SS-FBPI. These observations are consistent with the AA composition of the fava bean protein isolates (Table 7.3S-Appendix 25). Heat-stable attractions, such as hydrophobic interactions, are more favorable at higher temperatures due to their endothermic effect (Sun & Arntfield, 2012). Therefore, the formation of a gel structure during heating likely involves hydrophobic interactions. Upon cooling, the three-dimensional gel network may be further reinforced by the covalent (disulfide bridges) and other non-covalent bonds, such as hydrogen and electrostatic bonds. In particular, hydrogen bonds are weaker at higher temperatures and vice versa. The net effect of these molecular forces is believed to be crucial for maintaining the overall balance of attractive and repulsive forces, thereby contributing to the formation of a strong gel.

The driving force behind the dissolution of proteins in DES extraction is the formation of hydrogen bonds between the protein and DES components. In part, the ionic strength of the DES also plays an important role in the extraction process (neutral pH), thereby changing the

physiochemical properties of native proteins (Hewage et al., 2022). Similar to SE, the ionic effect of DES (choline and chloride) may involve an electrostatic shielding effect, non-specific charge neutralization, and direct ion-protein interactions (Zhang & Cremer, 2006). Our previous study (Hewage et al., 2024) confirmed that DES produced high-quality FBPI with lower denaturation. Therefore, it can be inferred that ions from DES primarily influence electrostatic interactions on the charged groups on the surface of proteins, rather than the buried core of the proteins. As a result, slightly higher ionic interactions also contributed to the formation of the gel structure. Compared to all other gels, the involvement of the covalent bonds (disulfide bridges) in DES-FBG formation was significantly higher ($P < 0.05$). Since DES extraction favored the higher legumin fraction over the AE and SE (Table 7.1), it can be expected that more disulfide linkages are involved during gelation [Figure 7.8(b)]. This is likely due to a lower electrostatic repulsion between the protein aggregates (Visschers & De Jongh, 2005).

Unlike DES extraction, SE depends completely on the ionic strength of the solvent. Significantly higher ionic bonds ($P < 0.05$) confirm that electrostatic bonds were directly associated with gel network formation, in addition to the principal hydrophobic interactions. The contribution from disulfide bonds, on the other hand, was lower ($P < 0.05$) compared to DES-FBG but higher ($P < 0.05$) than ALK-FBG. The effect could be due to the lower percentage of legumin fraction extracted than that of the DES-FBG, and more electrostatic interactions may have been favored over the disulfide linkages during the cooling stage [Figure 7.8(c)]. Sun & Arntfield (2010) reported that hydrophobic and hydrogen bonding interactions were the primary contributors to network formation in the salt-extracted pea proteins. In another study, the salt-extracted pea proteins showed a lower legumin ratio, which reduced the large insoluble aggregates that bind through disulfide bonds during heat-induced gelation (Yang et al., 2021). In this study, we observed

that the involvement of the hydrogen bonds in gelation was limited to all gels. However, ALK-FBG had significantly higher ($P < 0.05$) hydrogen bonds than other counterparts, but not in greater proportion. ALK-FBPI exhibited the lowest legumin ratio, indicating that a higher 7S fraction may have contributed to the reduction in disulfide bond formation between aggregates, leading to a poor gel structure. Moreover, as can be seen, fewer electrostatic interactions also facilitated the formation of the gel network of ALK-FBG [Figure 7.8(d)]. The reduction of electrostatic repulsion and ionic strength between the polar groups of protein molecules during gel formation might have driven the lower content of ionic bonds (Kohyama et al., 1995).

Overall, it can be summarized that hydrophobic interactions are the dominant forces maintaining the gel network of all gels prepared from different extraction methods. Subsequent gel strength was primarily reinforced by disulfide bonds, electrostatic interactions, and, to a lesser extent, hydrogen bonds. Therefore, this information is useful in explaining the textural changes of gels and can potentially be modified for desired textural attributes for specific food formulations.

7.4.3.4. Changes in gel hardness and water holding capacity (WHC)

The gel strength, as analyzed from the compression test using the texture analyzer, is shown in Figure 7.7(b) and Table 7.2S (Appendix 24). The mechanical properties are of primary importance in understanding the textural characteristics of food ingredients. In general, the rupture strength of the gels is expected to increase with more cross-links formed. However, maximum gel rigidity is achieved by the optimum balance between protein-protein interactions and protein-solvent interactions. Gels formed by ALK-FBPI and SS-FBPI were observed to be gritty and brittle, respectively, and exhibited very soft properties, easily changing shape. This made it difficult to perform the texture analysis. Therefore, only gels prepared from CS-PI and DES-FBPI were evaluated for their gel strength. The gel strength depends on the net effect of disulfide, ionic,

hydrophobic, and hydrogen bond interactions (Rahman & Lamsal, 2023). In addition, the gel firmness or strength may be dictated by hydrogen bonds and/or hydrophobic bonds (Shan et al., 2015). Since H-bond contribution from all gels was fairly low, a significant reduction of hydrophobic interactions among ALK-FBPI and SS-FBPI, compared to DES-FBPI and CS-PI, might be attributed to the poor gel network (Lin et al., 2019). However, disulfide bonds also profoundly affected the gel strength and firmness (Shan et al., 2015), as clearly demonstrated by the significantly higher ($P < 0.05$) gel hardness of DES-FBG (921.57 ± 49.43 g) compared to CSG (567.61 ± 89.04 g).

Visually, gels from the CS-PI produced a rubbery-like texture, consistent with previously reported data (Chronakis et al., 1995). Sometimes, the excessive formation of hydrophobic bonds can also result in inferior quality gels, characterized by their rubbery texture (Chen et al., 2021). In contrast, DES-FBG exhibited a tofu-like smooth appearance and texture, indicating that disulfide bonds were crucial in forming a firm gel network. It was observed that even the SS-FBG had a slightly firm texture and a smooth appearance. The effect may be due to the contribution of ionic and disulfide bonds in the gel network. On the contrary, ALK-FBG was shown to be a brittle structure with an uneven shape. The reason could be explained by the larger particle size of proteins evident by our previous research (Hewage et al., 2024), which caused the physical constraints to make a strong gel network. Moreover, protein oxidation can have a negative impact on the side chains of amino acids and the protein's structure, affecting its gelation properties (Nawaz et al., 2022). Oxidation may result in the loss of net charges and increased surface hydrophobicity of protein molecules, subsequently promoting greater random aggregation between proteins (Wanjun et al., 2024). Therefore, oxidation may form a weaker gel network in

ALK-FBPI, SS-FBPI, and CS-PI, compared to DES-FBPI, supported by the XPS O1s spectral analysis.

The water-holding capacity (WHC), another important property of the gel network, plays a crucial role in food processing and the development of gel-based foods. It provides insights into understanding the effects of protein aggregates on WHC and helps identify the textural properties of gels. As illustrated in Figure 7.7(c), all gels represented fairly better water WHC above 75%; The highest WHC ($P < 0.05$) was observed in CSG (101.21%), followed by ALK-FBG (95.67%), DES-FBG (81.82%), and SS-FBG (75.45%). The same trend was observed with the WHC of these protein isolates in our previous study (Chapter 4), indicating that the degree of protein denaturation during the extraction process may be directly correlated with the WHC of gels. The study by Bühler et al. (2020) demonstrated that dry-heated (75-175 °C) faba bean protein concentrate yielded higher WHC, achieving a value comparable to that of soy protein concentrate. They suggested that these changes were due to partial protein denaturation, which alters the protein structure and exposes hydrophobic sites. The microstructure of the protein matrix can also determine the water-binding capacity (Lam et al., 2018). Arrese et al. (1991) reported that a high degree of denatured soy proteins had higher water imbibing capacity than the native-state soy protein isolates. In addition, the larger size of the protein aggregates of CSG may influence the compact gel network, trapping more water (Wang et al., 2017). Furthermore, the reduced water-holding capacity (WHC) of SS-FBG may be due to the coarseness of the gel network (Figure 7.8), with larger aggregates, which is in agreement with the findings of Wu et al. (2019). More uniform gel networks with higher strength in DES-FBG retain a significant amount of water; however, the rate of aggregation and their size during heating may govern the WHC.

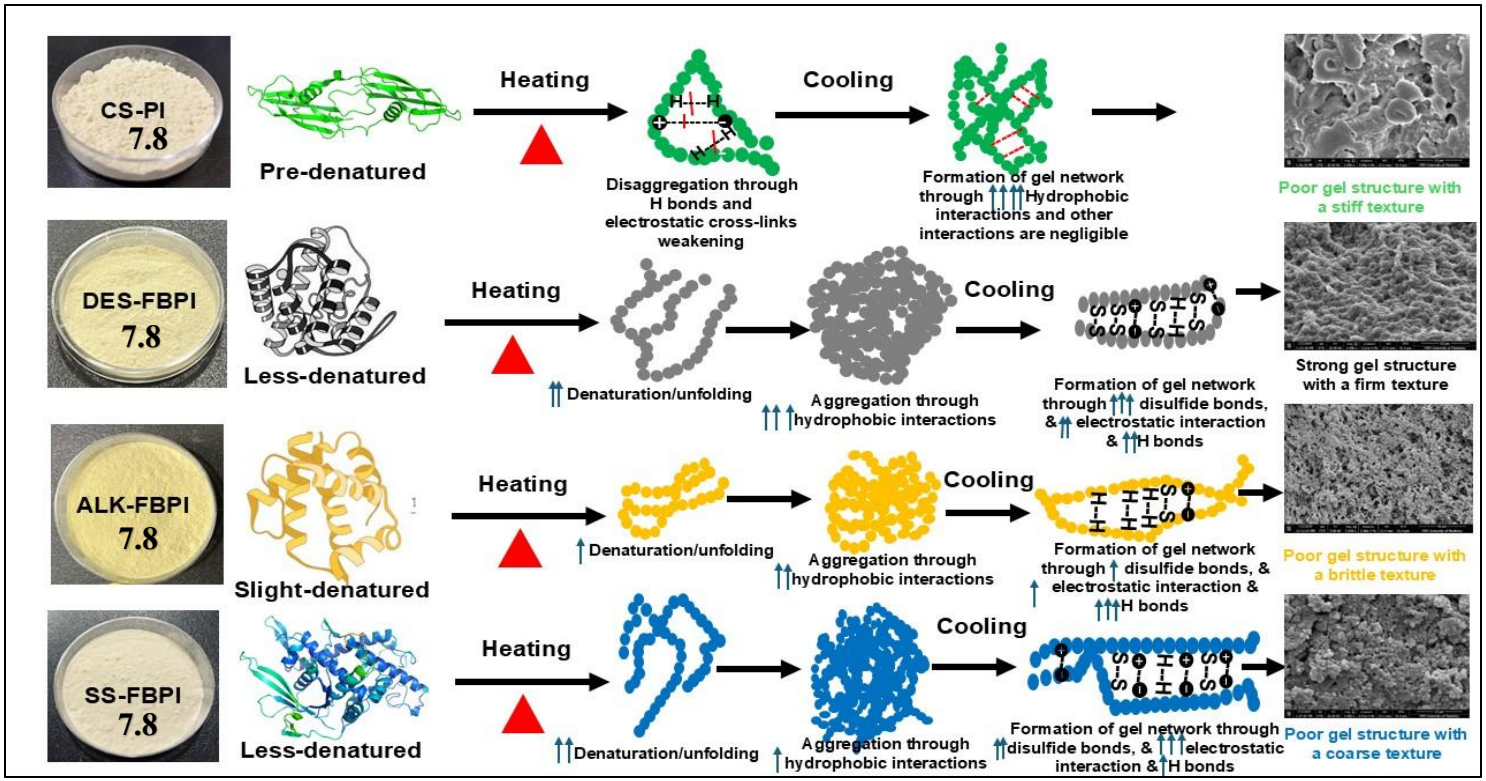


Figure 7. 8. Gel formation mechanism and molecular interaction of protein isolates extracted by various methods, *i.e.,* **(a)**-Commercial soy protein isolates (CS-PI), **(b)**-Deep eutectic solvent-extracted fava bean protein isolates (DES-FBPI), **(c)-** Alkaline-extracted fava bean protein isolates (ALK-FBPI), and **(d)**-Salt-extracted fava bean protein isolates (SS-FBPI). ▲-Represent the heating and arrows indicate the levels, ↑- low, ↑↑- medium, and ↑↑↑-higher.

7.4.3.5. Microstructure of gels

The morphology of gels prepared from the respective protein isolates, characterized by SEM, is depicted in Figure 7.9. SEM images revealed that gels prepared from ALK-FBPI and SS-FBPI were composed of irregular protein aggregates loosely stacked, with larger pores. Thus, ALK-FBPI and SS-FBPI can be considered as particulate gels. In contrast, DES-FBG showed homogenous, well-connected, fine-stranded, smaller pores and polymer-like gels with many thick junction zones. It has been demonstrated that fine-stranded gels are much more elastic than particulate gels (Nieto-Nieto et al., 2014), which agrees with our findings. The right balance between hydrophobic and repulsive forces among the polypeptide chains of DES-FBPI may have likely allowed the formation of bridges between protein-protein interaction points. In addition, the higher disulfide cross-links between the aggregated DES-FBPI may have reinforced the gel network, imparting strong mechanical properties [Figure 7.7(b)].

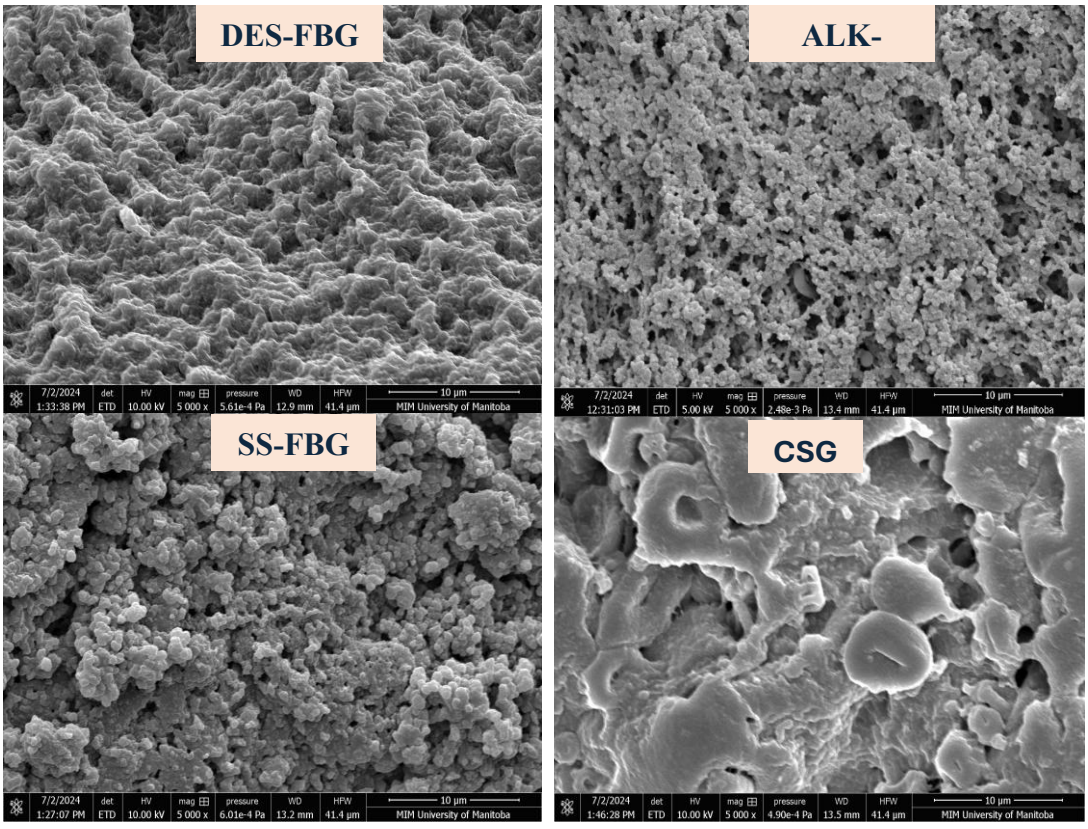


Figure 7. 9. Scanning electron microscopy (SEM, ×5000) of gel images prepared (at 20% (w/v) protein concentration and pH 7.0) by proteins extracted by various methods, *i.e.,* Deep eutectic solvent-extracted fava bean gels (DES-FBG), Alkaline-extracted fava bean gels (ALK-FBG), Salt- extracted fava bean gels (SS-FBG), and (e)-Commercial soy protein gels (CSG).

This can be further explained by the 11S/7S ratio; DES-FBPI had a higher ratio (2.71) than both SS-FBPI (2.32) and ALK-FBPI (1.48). The gelling behavior of 11S was reported to be superior to that of the 7S proteins in fava beans (Oluwajuyitan & Aluko, 2024), attributed to the presence of cysteine residues in the legumin fraction. However, vicilin lacks cysteine residues and aggregates primarily through non-covalent interactions (Mession et al., 2015), resulting in small aggregates (O'Kane et al., 2005). Since the FBPI produced from the AE had a higher proportion of 7S fractions (with the lowest 11S/7S ratio, 1.48), the corresponding gels exhibited smaller aggregates, forming a particulate structure. This has likely limited the interaction between large aggregates via disulfide bonds, as confirmed by the significantly lower G' value throughout the temperature sweep test. The Pore size of ALK-FBG was even larger and had a thin, limited number of junction zones. Similarly, SS-FBG had larger pores, but protein aggregates were much larger, coarser, and irregular, with no visible junction zones. Larger aggregates may have facilitated more disulfide cross-links (11S/7S=2.32) between them. However, the higher rate of aggregate formation by SS-FBG [Figure 7.6(a)] during the temperature sweep (with a holding temperature of 90 °C for 30 min) may result in excessive attractive forces or insufficient charge repulsion, rather than proper network formation (Uruakpa, 2012). In the case of CSG, the gel formation from CS-PI appears to be rather complex, as various stages of aggregation and heterogeneous degrees of denaturation conditions are involved in the gelling process, unlike native proteins. It produced dense, heterogeneous, and disordered aggregates of much larger size. The gel texture

was rather rubbery; however, the outstanding self-supporting structure, compared to all gels, was a distinguishing feature of soy proteins, despite the lower G' than the DES-FBG and even the SS-FBG during the temperature sweep. Due to the high temperature in spray drying, CS-PI is often partially denatured. This was evidenced by their higher surface hydrophobicity, larger particle sizes, reduced protein solubility (Chapter 2), and lower tryptophan fluorescence [Figure 7.3(a)]. Due to the high surface hydrophobicity of CS-PI and pre-aggregation, less thermal energy is required to dissociate subunits, thereby forming dense domain particles. This process could facilitate the development of a vigorous gel microstructure with increased stiffness (Chen et al., 2017). Therefore, the abundance of pre-aggregated proteins might be the reason for producing the compact gel microstructure and hardened gel by CS-PI. Similar observations were reported by Zhang et al. (2025), who evaluated 14 varieties of CS-PI to investigate differences in gelation. According to their findings, pre-denatured soy protein isolates exhibited the largest extent of protein oxidation and formation of hard gels compared to their hydrolyzed counterparts, indicating that pre-aggregated proteins were responsible for the stiff gel formation.

7.5. Conclusion

This study aimed to investigate the effect of DES extraction on the structure and gelling properties of fava bean protein compared to conventional extraction methods. CS-PI served as a reference material due to its wide applicability as a benchmark for comparing other plant protein gel systems. This study demonstrated that DES-FBPI exhibited a lower degree of protein denaturation and oxidation compared to conventional methods, which may provide insight into using DES as an alternative, sustainable method to its conventional counterparts. The development of the gel structure of DES-FBG was different from that of ALK-FBG, SS-FBG, and CSG. Notably, these differences contributed to a higher 11S/7S ratio in DES-extracted fava bean proteins and increased

involvement of sulfur-containing amino acids, particularly cysteine residues, in gel formation. Molecular interactions showed that all gels were predominantly stabilized by hydrophobic interactions. DES-FBPI had significantly higher disulfide bonds in the gel matrix than in all other cases, which agreed with the formation of a stronger gel network. The DES-FBPI behaved fundamentally differently than CS-PI, resulting in a strong, viscoelastic gel network that may offer opportunities for novel protein-rich food formulations compared to soy-derived protein sources, as an allergen-free paradigm. Therefore, this study highlights the potential application of DES extraction on an industrial scale to expand sustainable protein ingredient production. The generated knowledge will be useful for both industry and academia in further investigating the gelling properties of fava bean protein.

Chapter 8

General Discussion, Conclusion, and Recommendations

8.1. General Discussion

Although fava beans are rich in nutrients, including starch, protein, fiber, and bioactive compounds, their consumption in human diets is relatively low compared to other pulses, such as soybeans and peas. However, the recent deliberate research on protein ingredient production from fava beans brings a gradual spotlight to the protein ingredient market with innovative product lines. The recent surge in demand for sustainable green production among consumers has posed significant challenges to the food industry, compelling it to shift from conventional practices to a more eco-friendly paradigm in protein ingredient production. The poor protein functionality and environmental hazards associated with conventional protein extraction methods have driven extensive exploration of novel, sustainable techniques in contemporary food processing and ingredient production (Hewage et al., 2022). Research into protein extraction from DES has increased significantly in recent years but has yet to demonstrate the improved protein yield and content of many sources comparable to those of conventional alkaline extraction (Hewage et al., 2024). Our research, which utilized the systematic optimization method achieved through the RSM model, indicated a high protein yield and protein content in fava beans, surpassing those of conventional extraction methods. Most of the previously reported data on DES protein extraction and the optimization of extraction variables had not been taken into account, resulting in relatively low protein yield and content (Grudniewska et al., 2018; Yue et al., 2022). Optimizing the DES system for a specific plant source is crucial, as the interaction between DES components and protein molecules varies based on the protein's molecular structure (Sanchez-Fernandez & Jackson, 2021). The protein structure and the varied DES components may interact with proteins differently. Therefore, optimization is a must for achieving a high protein yield and protein content.

Although alkaline extraction depends on a higher pH adjustment, DES containing ChCl and Gly did not require pH adjustment during the extraction. Despite the DES having a slightly acidic pH after synthesis, the protein extraction at 50 °C automatically brought the pH to neutral and maintained it throughout the extraction process. Thus, unlike alkaline extraction, DES acted as a buffer that could be greatly advantageous in producing less denatured proteins. Additionally, the molar ratio of ChCl and Gly (1:2) obtained from optimization may substantially affect the buffering action of the DES system. The hydrogen bond donor, Gly, is the key component that governs the acidity of the system (Skulcova et al., 2018); the lower molar ratio probably caused a mild impact on the protein conformation, leading to lower denaturation. This may have a subsequent positive impact on protein functionality, as observed in this study. Although the interaction of DES components with protein molecules is not yet well understood in research, the driving force behind DES, the involvement with hydrogen bonds, may induce different conformational changes in proteins (Sanchez-Fernandez & Jackson, 2021), leading to improved protein functionality.

Approximately 50% of the protein extraction efficiency with the optimized DES system indicates that the remaining half is discarded as insoluble materials. Vigorous destruction of the plant cells is necessary to increase extraction efficiency further. The pre-treatment employed offered considerable advantages in enhancing protein extraction efficiency. The optimization of ultrasonication (US) provides insights into the optimal conditions for extracting the highest protein yield. The high amplitude and longer duration of the US pre-treatment would be detrimental to protein structure, leading to denaturation due to the high energy released by the cavitation process (Rahman & Lamsal, 2021). Therefore, the medium-level amplitude and shorter treatment time would be ideal for breaking the cell wall and liberating more proteins into the extraction medium.

The optimized conditions, which achieved an amplitude of 62% and a treatment time of 7 min, were sufficient to increase the protein yield by ~27% compared to the control, indicating their applicability in industrial applications. Nonetheless, protein purity may be affected when other cell constituents are released with the disruption of the cell wall, which was observed in this study. As a result, techno-functional properties can be negatively affected, for example, emulsification and foaming properties (Chang et al., 2022). Although the improved techno-functional properties, including solubility, water and oil holding capacity, and gelling capacity, were evident in our study, no improvements were observed in the emulsification and foaming properties. However, the increased particle size resulting from the US treatment of fava bean proteins compared to the control may also have impacted the interfacial properties. Even a 62% amplitude may be strong enough to alter the protein conformation and form free radicals; hence, careful selection of the US amplitude is crucial in the pre-treatment employed in the subsequent DES protein extraction. Additionally, since the US treatment was conducted in the DES environment, the ultrasound wave propagated through the medium, which could potentially alter the DES nanostructure, imparting varied hydrogen bonds and electrostatic forces between protein molecules. This would ultimately alter the protein conformation, and we still need to investigate this in-depth, as no research has been conducted in this regard. On the other hand, it is anticipated that US treatment in an aqueous environment (pure water) and subsequent DES extraction would likely yield outcomes different from those obtained in the current study.

Another pre-treatment method employed in this study for cell disruption was enzymatic pre-treatment (EP). Optimizing the EP conditions is also necessary to determine the optimal condition for maximum protein extraction through cell wall breakage. The pH, enzyme concentration, and temperature need to be optimized for optimal enzymatic hydrolysis of the cell

wall. The viscozyme L enzyme, a cocktail of carbohydrases, facilitates the efficient breakdown of various cell components, including cellulose, hemicellulose, pectin, xyloglucan, and arabinoxylan. From a protein functionality perspective, EP is more suitable for pre-treatment than US, as proteins remain unaffected and intact during hydrolysis. EP significantly altered the volume-weighted particle size of the protein by 2-fold compared to the control. This means that EP reduced the particle size 4 times lower than the US method. Thus, the impact on interfacial properties was not improved with the smaller particle size of the proteins, which probably failed to rearrange at the interface and made a strong viscoelastic film around the oil and air droplets despite the significantly higher solubility and the protein content (>95%) of EP-produced proteins compared to both the US and control samples. It is unclear why the EP produced a very small particle size; however, the aqueous enzymatic hydrolysis performed under rigorous stirring conditions, coupled with subsequent DES extraction, may have ultimately affected the particle size of proteins. Enzyme activity under a DES environment is an interesting topic, allowing for a significant reduction in pre-treatment time compared to the initial aqueous (pure water) treatment and subsequent DES extraction.

The viscosity of DES is a primary barrier to industrial application. Even under optimized DES extraction conditions, with a 40% w/w content, the solvent remains slightly viscous. Viscosity may also have affected the pre-treatment process, lowering the enzyme diffusion and US wave propagation through the solvent media. Substitution of water with DES at varied percentages provides novel insights into how the protein conformation and the functional properties change, corresponding to its physiochemical properties and nanostructure alterations. Since DES are complex solvents formed between hydrogen bond donors and acceptors, their physical properties vary upon the addition of water, resulting in different interactions between the protein and DES

components (Zahn et al., 2016). Thus, protein microenvironment can be modified through its composition. This may alter the balance of molecular forces, including hydrogen bonds, electrostatic forces, and van der Waals forces in the protein's amino acids, to that of the different water content in DES, resulting in various protein structures and conformations. This was clearly demonstrated by the surface hydrophobicity (Figure 6.5), FTIR (Table 6.5), and SDS-PAGE (Figure 6.6) results obtained in this study. Adding water from 0% to 80% w/w significantly reduced the viscosity of DES, thereby enhancing the protein yield from ~29% to 84%. In part, adding water plays a key role in the solvation of protein molecules. This represents a tremendous increase in protein yield, and interestingly, the protein content achieved was ~90%, even at an 80% water addition. From an industrial perspective, this would be the biggest advantage in terms of protein purity and reduced chemical consumption. Based on the NMR and the FTIR data on the DES structural changes observed with the varied water content, the identified three transitions points, (1) 0-10 wt% water (water in DES) where structural integrity is fully maintained, (2) 10-40 wt% water (water in DES) where structural integrity is partially distorted and (3) >40 wt% water (DES in water) where the structure is completely distorted and behave as an aqueous solution. Corresponding to each of these phases, the protein functionality differed due to the different protein conformations. Therefore, the tunability of DES enables industries to design a specific “designer” DES system for extracting protein tailored to a specific application. However, the behavior of plant protein and its molecular dynamics in the DES-water system is scarce and should be well-prioritized in research for a deeper understanding of developing novel technologies for protein-based functionalities. Especially, in the “DES in water” system, where the DES structure is completely distorted and behaves as an aqueous solution, the protein behavior remains unexplored.

Nonetheless, our study may serve as an initial benchmark for future investigations and provide basic knowledge on the behavior of plant proteins in DES.

Overall, the ultimate goal of this study is to industrialize the production of fava bean protein ingredients using the DES technique for application in food products. The one distinguishing feature of the protein functionality in the extracted fava bean protein was its gelling capacity, which is comparable to commercial soybean proteins. Due to the limited scientific knowledge of the gelling behavior of DES-extracted proteins, our goal was to investigate the protein's surface characteristics, rheological properties, and molecular interactions in gels and to elucidate the underlying gelling mechanism. The remarkable complexity of protein behavior in the DES environment requires an in-depth investigation to study the gelling characteristics, enabling the fava bean protein to be positioned in the market comparable to soybean-based products. The conventionally produced fava bean protein isolates, obtained through salt and alkaline extraction, and the commercial soybean protein exhibited higher oxidation (XPS analysis - Appendix 25) than the DES extraction. Protein oxidation is believed to play a causative role in the textural quality of foods (Xiong & Guo, 2020). The most notable result of protein oxidation is the loss of side-chain amino acids, particularly sulfur-containing ones (Xiong & Guo, 2020). The oxidation could further induce the random aggregation of proteins and affect the formation of intermolecular interactions, such as covalent bonds (S-S) between protein aggregates, thereby resulting in a poor gel structure, which is probably what we observed with the conventional extraction techniques. However, mild oxidation is reported to promote protein-protein interactions with improved gel elasticity, firmness, and strength (Zhou et al., 2014), which is consistent with the results obtained for DES extraction. Therefore, it is worth noting that although we did not measure the oxidation levels of the extracted proteins, the surface properties determined by XPS provided substantial information on the quality

of the proteins. However, it is warranted that the protein oxidation of extracted protein isolates be evaluated to understand the correlation between protein functionality better. Moreover, DES extraction favored higher legumin fractions over the other method employed, resulting in a higher proportion of sulfur-containing amino acids in the isolates. This was associated with better covalent cross-links in gel formation, contributing to a firmer gel structure than conventional counterparts. Commercial soybean proteins appear to have a different gelation mechanism, accompanied by pre-aggregation induced by commercial processing; however, in essence, DES extraction yielded promising findings for novel food applications with its remarkable gelling capacity. With the recent introduction of a novel product line featuring fava bean tofu and consumer-facing snack products in the Canadian market, we are now poised to drive the use of fava beans, leveraging our novel findings, in a variety of food formulations in the future. Therefore, our study would further advance research in the Canadian protein industry by initiating new partnerships to enhance the value chain of fava beans.

8.2. General Conclusion

This research aimed to develop a sustainable extraction technology using deep eutectic solvents (DES) to improve the functionality of fava bean protein isolates. The industrial scalability of a novel protein extraction method depends on optimizing key variables to achieve high protein yield, purity, and quality. This remains a significant challenge for emerging extraction technologies intended for large-scale production. In this study, a DES system composed of choline chloride and glycerol successfully extracted fava bean protein isolates, with process optimization conducted using response surface methodology. The protein yield was significant, with approximately 65% obtained compared to conventional alkaline extraction (~60%), and the protein content was approximately 92% on a dry basis. Generally, the industrial method, the alkaline extraction,

achieves approximately 50% of protein yield with >90% protein purity; hence, DES extraction has the potential to be industrialized on a large scale. To accomplish the scale-up process, the protein's functionality and quality must stand out as superior attributes to apply in novel food formulations and food processing. Compared to conventional methods, DES extraction produced protein isolates with improved techno-functional properties, which are beneficial in novel food formulations, leading to the selection of healthy and sustainable protein options for Canadians. Especially for consumer-facing products, particularly snack foods, fava bean protein presents a tremendous opportunity for Canada's food and agriculture sector. The unique feature of the DES-extracted fava bean protein isolates (DES-FBPI) was their superior gelling properties, which were rated far beyond those of commercial soy protein isolates. The firm, smooth, and strong gel texture of DES-FBPI would be a potential hallmark for positioning fava beans in the competitive protein ingredient market, such as in tofu, compared to soybean protein. The value of these new potential food processing and formulations would further augment the quality of the protein. The DES-FBPI produced high-quality protein with high amounts of essential amino acids, particularly sulfur-containing amino acids, and higher (>75%) *in vitro* protein digestibility-corrected amino acid score (PDCAAS), which probably amplifies the innovation in the realm of fava beans as a value-added food item. Combining DES extraction with other novel technologies, such as ultrasonication and enzymatic pre-treatment, as an integrated approach, further significantly increased the protein yield of FBPI, with substantial improvements in functional properties, including solubility, water-holding capacity, and fat-holding capacity. Notably, the gelling capacity of pre-treated FBPI exhibited lower gel concentration at a wider pH range. This suggests that the protein has been modified by pre-treatment methods, which could further enhance its suitability for food applications. It is noteworthy that enzymatic pre-treatment was capable of producing high-purity

protein (~97%) with a significantly reduced volume-weighted particle size (<15 μm) and without a fat component, which makes it a promising candidate for plant-based beverages and the development of protein-based nano-delivery systems. Despite the aforementioned innovations, the high viscosity of DES remains a significant barrier to industrial applications. Water addition to DES is inevitable in reducing viscosity, and our study showed that drastic changes in the physiochemical parameters of different DES-water systems could be tailored to design specific DES systems for the specific functionality of the protein. This means that the amount of water can tune the DES structure with its altered physical properties to tailor-made protein functional properties. This is a distinguishing property of DES compared to the other conventionally used organic solvents in protein extraction. Thus, DES extraction would be ideal for innovative work in the protein ingredient market. This was clearly indicated by the superior gelling capacity and the strong gel network of the DES-FBPI compared to conventionally produced FBPI and the commercial soy proteins. Therefore, innovation in DES protein extraction likely opens up a diverse array of opportunities to replace soy protein-based gel products, which hinder market expansion for protein ingredients due to serious concerns about allergens. This may position fava bean protein as a more versatile and acceptable option than soy proteins, further enhancing its growth prospects in the protein ingredient market.

Overall, the knowledge and data generated in this research provide valuable insights for crop breeders, ingredient manufacturers, and food scientists, laying the groundwork for capturing the growing global protein opportunity while enhancing environmental sustainability by offering healthy options to consumers. Moreover, the novel findings of this project further expand the cooperation across the value chain to bring high-quality protein ingredients to Canadian-made products by encouraging the domestic cultivation and processing of fava beans. Ultimately,

bolstering the innovative and sustainable approaches demonstrated in this research to enhance the processing of fava beans for value-added protein ingredients will provide opportunities to advance cutting-edge research in the protein industry and establish stronger fava bean value chains, leading to a greater economic return.

8.3. Limitations of the Study

This research study has several limitations.

1) The DES component, choline chloride, was highly hygroscopic in nature, so during DES preparation, moisture absorption from the surroundings was unavoidable.
2) Protein recovery from the DES consumes considerable time via the dialysis process due to the high viscosity of DES.
3) Some data analysis (amino acid analysis) was performed without replicates, as the associated costs were quite high.
4) Molecular dynamics simulations between the protein and DES were intended to be conducted, but the unavailability of computational equipment for molecular simulations prevented the test.
5) The residual effect of DES and the animal model's toxicity test of protein isolates was discarded due to time constraints.

8.4. Recommendation for future research

Protein extraction using DES is still in its early stages, and this is the first research study to explore protein extraction using DES and the behavior of proteins with DES through an in-depth analysis. However, in addition to our findings, fundamental aspects of DES and protein interactions still need to be investigated. Therefore, the following recommendations are warranted,

1) Further investigation should focus on fava bean proteins, which may be compatible with other DES systems beyond those studied in this research, to explore protein yield and possible interactions between the protein and solvent.
2) We considered only one fava bean variety, Snowbird (tannin-free), grown in Canada for protein extraction using the DES system and optimization, as our primary focus was on developing the extraction technology. Therefore, future investigations are warranted for the other fava bean varieties to determine changes in protein yield and techno-functional properties for broader food applications.
3) Experimental methods often provide limited observations of the molecular and solvation interactions. Therefore, computational approaches such as molecular dynamics (MD) simulation studies are necessary to investigate protein-solvent and solvent-solvent interactions, which can subsequently relate to improved protein function.
4) Though our study investigated the *In-vitro* PDCASS, the digestibility study using a simulated digestion method, representing the oral, gastric, and intestinal phases, is required to understand the digestion behavior of DES-FBPI for the novel food formulations.
5) Despite the non-toxicity of choline chloride and glycerol, the residual effect of DES-FBPI should be assessed to confirm the safety of the protein ingredient before it is used in novel food processing.
6) A toxicological assessment of DES-FBPI is crucial for establishing an efficient extraction method on an industrial scale, which will facilitate subsequent development of food products.

7) The recyclability efficiency of the choline chloride and glycerol DES system should be explored for better re-utilization of chemicals to enhance the economic feasibility of this extraction method.
8) Technology scale-up, such as pilot-scale testing, is necessary to evaluate the DES protein extraction process, including the pumping system, protein yield, and recovery, before expanding to an industrialized larger scale.

References

Abbott, A. P., Capper, G., Davies, D. L., Rasheed, R. K., & Shikotra, P. (2005). Selective extraction of metals from mixed oxide matrixes using choline-based ionic liquids. *Inorganic Chemistry, 44*(19), 6497-6499.

Abbott, A. P., Capper, G., Davies, D. L., Rasheed, R. K., & Tambyrajah, V. (2003). Novel solvent properties of choline chloride/urea mixtures. *Chemical Communications*, *1,* 70–71.

Abdel-Aal, E. M., Shehata, A. A., El-Mahdy, A. R., & Youssef, M. M. (1986). Extractability and functional properties of some legume proteins isolated by three different methods. *Journal of the Science of Food and Agriculture*, *37*(6), 553–559.

Abranches, D. O., Silva, L. P., Martins, M. A. R., & Coutinho, J. A. P. (2021). Differences on the impact of water on the deep eutectic solvents betaine/urea and choline/urea. *Journal of Chemical Physics*, *155*(3).

Adetunji, L. R., Adekunle, A., Orsat, V., & Raghavan, V. (2017). Advances in the pectin production process using novel extraction techniques: A review. *Food Hydrocolloids, 62*, 239-250.

Aguilar-Acosta, L. A., Serna-Saldivar, S. O., Rodríguez-Rodríguez, J., Escalante-Aburto, A., & Chuck-Hernández, C. (2020). Effect of ultrasound application on protein yield and fate of alkaloids during lupin alkaline extraction process. *Biomolecules, 10*(2), 292.

Aissaoui, T. (2015). Pharmaceutica Analytica Acta Novel Contribution to the Chemical Structure of Choline Chloride Based Deep Eutectic Solvents. *Pharmaceutica Analytica Acta*, 6(11), 1000448.

Alashi, A. M., Blanchard, C. L., Mailer, R. J., Agboola, S. O., Mawson, A. J., He, R., Malomo, S. A., Girgih, A. T., & Aluko, R. E. (2014). Blood pressure lowering effects of Australian canola protein hydrolysates in spontaneously hypertensive rats. *Food Research International*, *55*, 281–287.

Alavi, F., Chen, L., & Emam-Djomeh, Z. (2021). Effect of ultrasound-assisted alkaline treatment on functional property modifications of faba bean protein. *Food Chemistry*, *354*.

Alavi, F., Chen, L., Wang, Z., & Emam-Djomeh, Z. (2021). Consequences of heating under alkaline pH alone or in the presence of maltodextrin on solubility, emulsifying and foaming properties of faba bean protein. *Food Hydrocolloids*, *112*, 106335.

Ali, F., Ippersiel, D., Lamarche, F., & Mondor, M. (2010). Characterization of low-phytate soy protein isolates produced by membrane technologies. *Innovative Food Science and Emerging Technologies, 11*(1), 162-168.

Alomar, M. K., Hayyan, M., Alsaadi, M. A., Akib, S., Hayyan, A., & Hashim, M. A. (2016). Glycerol-based deep eutectic solvents: Physical properties. *Journal of Molecular Liquids*, *215*, 98–103.

Alonso-Miravalles, L., Jeske, S., Bez, J., Detzel, A., Busch, M., Krueger, M., Wriessnegger, C. L., O'Mahony, J. A., Zannini, E., & Arendt, E. K. (2019). Membrane filtration and isoelectric precipitation technological approaches for the preparation of novel, functional and sustainable protein isolate from lentils. *European Food Research and Technology 2019 245:9*, *245*(9), 1855–1869.

Alonso, R., Aguirre, A., & Marzo, F. (2000). Effects of extrusion and traditional processing

methods on antinutrients and in vitro digestibility of protein and starch in faba and kidney beans. *Food Chemistry*, *68*(2), 159–165.

Altuner, E. M., Çeter, T., & Alpas, H. (2012). High hydrostatic pressure processing: A method having high success potential in pollen protein extraction. *High Pressure Research, 32*(2), 291-298.

Amagliani, L., Silva, J. V. C., Saffon, M., & Dombrowski, J. (2021). On the foaming properties of plant proteins: Current status and future opportunities. *Trends in Food Science & Technology*, *118*, 261–272.

Amiri, A., Sharifian, P., & Soltanizadeh, N. (2018). Application of ultrasound treatment for improving the physicochemical, functional and rheological properties of myofibrillar proteins. *International Journal of Biological Macromolecules*, *111*, 139–147.

AOAC. (2000). Association of Official and Analytical Chemist. Official method of Analysis. Official Method of Analysis, Washington, D*C.*

AOAC International. (2012). *Official methods of analysis of AOAC International* (G. W. Latimer (ed.); 19th ed.). AOAC International.

Arrese, E. L., Sorgentini, D. A., Wagner, J. R., & Añón, M. C. (1991). *Food Chemistry*. *39*, 1029–1032.

Arrutia, F., Binner, E., Williams, P., & Waldron, K. W. (2020). Oilseeds beyond oil: Press cakes and meals supplying global protein requirements. *Trends in Food Science & Technology*, *100*, 88–102.

Aryee, A. N. A., Agyei, D., & Udenigwe, C. C. (2018). Proteins in Food Processing. In R.Y. Yada (Eds.), *Impact of processing on the chemistry and functionality of food proteins* (pp.27–45). Woodhead Publishing.

Arzeni, C., Martínez, K., Zema, P., Arias, A., Pérez, O. E., & Pilosof, A. M. R. (2012). Comparative study of high intensity ultrasound effects on food proteins functionality. *Journal of Food Engineering*, *108*(3), 463–472.

Aschemann-Witzel, J., Gantriis, R. F., Fraga, P., & Perez-Cueto, F. J. A. (2021). Plant-based food and protein trend from a business perspective: markets, consumers, and the challenges and opportunities in the future. *Critical Reviews in Food Science and Nutrition*, 1–10.

Assatory, A., Vitelli, M., Rajabzadeh, A. R., & Legge, R. L. (2019). Dry fractionation methods for plant protein, starch and fiber enrichment: A review. *Trends in Food Science and Technology, 86*, 340-351.

Atefi, E., Fyffe, D., Kaylan, K. B., & Tavana, H. (2016). Characterization of aqueous two-phase systems from volume and density measurements. *Journal of Chemical and Engineering Data, 61*(4), 1531-1539.

Augustin, M. A., & Cole, M. B. (2022). Towards a sustainable food system by design using faba bean protein as an example. *Trends in Food Science & Technology*, *125*, 1–11.

Awad, T. S., Moharram, H. A., Shaltout, O. E., Asker, D., & Youssef, M. M. (2012). Applications of ultrasound in analysis, processing and quality control of food: A review. *Food Research International*, *48*(2), 410–427.

Awaluddin, S. A., Thiruvenkadam, S., Izhar, S., Hiroyuki, Y., Danquah, M. K., & Harun, R. (2016). Subcritical water technology for enhanced extraction of biochemical compounds from *Chlorella vulgaris*. *BioMed Research International, 2016*, 5816974.

Badjona, A., Bradshaw, R., Millman, C., Howarth, M., & Dubey, B. (2024). Structural, thermal, and physicochemical properties of ultrasound-assisted extraction of faba bean protein isolate (FPI). *Journal of Food Engineering*, *377*, 112082.

Banerjee, S., & Bhattacharya, S. (2012). Food Gels: Gelling Process and New Applications. *Critical Reviews in Food Science and Nutrition*, *52*(4), 334–346.

Bangar, S. P., Esua, O. J., Sharma, N., & Thirumdas, R. (2022). Ultrasound-assisted modification of gelation properties of proteins: A review. *Journal of Texture Studies*, *53*(6), 763–774.

Barac, M., Cabrilo, S., Pesic, M., Stanojevic, S., Zilic, S., Macej, O., & Ristic, N. (2010). Profile and Functional Properties of Seed Proteins from Six Pea (Pisum sativum) Genotypes. *International Journal of Molecular Sciences*, *11*(12), 4973.

Barba, F. J., Galanakis, C. M., Esteve, M. J., Frigola, A., & Vorobiev, E. (2015). Potential use of pulsed electric technologies and ultrasounds to improve the recovery of high-added value compounds from blackberries. *Journal of Food Engineering, 167*, 38-44.

Barth, A. (2007). Infrared spectroscopy of proteins. *Biochimica et Biophysica Acta (BBA) - Bioenergetics*, *1767*(9), 1073–1101.

Batista, A. P., Portugal, C. A. M., Sousa, I., Crespo, J. G., & Raymundo, A. (2005). Accessing gelling ability of vegetable proteins using rheological and fluorescence techniques.

International Journal of Biological Macromolecules, *36*, 135–143.

Bedin, S., Netto, F. M., Bragagnolo, N., & Taranto, O. P. (2020). Reduction of the process time in the achieve of rice bran protein through ultrasound-assisted extraction and microwave-assisted extraction. *Separation Science and Technology, 55*(2), 300-312.

Behere, M., Patil, S. S., & Rathod, V. K. (2021). Rapid extraction of watermelon seed proteins using microwave and its functional properties. *Preparative Biochemistry and Biotechnology, 51*(3), 252-259.

Benelhadj, S., Gharsallaoui, A., Degraeve, P., Attia, H., & Ghorbel, D. (2016). Effect of pH on the functional properties of Arthrospira (Spirulina) platensis protein isolate. *Food Chemistry*, *194*, 1056–1063.

Berghout, J. A. M., Boom, R. M., & van der Goot, A. J. (2015). Understanding the differences in gelling properties between lupin protein isolate and soy protein isolate. *Food Hydrocolloids*, *43*, 465–472.

Bhattarai, R. R., Dhital, S., Mense, A., Gidley, M. J., & Shi, Y. C. (2018). Intact cellular structure in cereal endosperm limits starch digestion in vitro. *Food Hydrocolloids*, *81*, 139–148.

Bi, X., Hemar, Y., Balaban, M. O., & Liao, X. (2015). The effect of ultrasound on particle size, color, viscosity and polyphenol oxidase activity of diluted avocado puree. *Ultrasonics Sonochemistry*, *27*, 567–575.

Bleakley, S., & Hayes, M. (2017). Algal proteins: Extraction, application, and challenges concerning production. *Foods, 6*(5), 33.

Boukid, F., & Castellari, M. (2022). How can processing technologies boost the application of faba bean (Vicia faba L.) proteins in food production? *EFood*, *3*(3), e18.

Boye, J., Zare, F., & Pletch, A. (2010a). Pulse proteins: Processing, characterization, functional properties and applications in food and feed. *Food Research International, 43*(2), 414-431.

Boye, J. I., Aksay, S., Roufik, S., Ribéreau, S., Mondor, M., Farnworth, E., & Rajamohamed, S. H. (2010). Comparison of the functional properties of pea, chickpea and lentil protein concentrates processed using ultrafiltration and isoelectric precipitation techniques. *Food Research International*, *43*(2), 537–546.

Bryant, S. J., Christofferson, A. J., Greaves, T. L., Mcconville, C. F., Bryant, G., & Elbourne, A. (2021). Bulk and interfacial nanostructure and properties in deep eutectic solvents: Current perspectives and future directions. *Journal of Colloid and Interface Science*, *608,* 2430-2454.

Bühler, J. M., Dekkers, B. L., Bruins, M. E., & Van Der Goot, A. J. (2020). Modifying Faba Bean Protein Concentrate Using Dry Heat to Increase Water Holding Capacity. *Foods*, *9*(8), 1077.

Byanju, B., Rahman, M. M., Hojilla-Evangelista, M. P., & Lamsal, B. P. (2020). Effect of high-power sonication pretreatment on extraction and some physicochemical properties of proteins from chickpea, kidney bean, and soybean. *International Journal of Biological Macromolecules*, *145*, 712–721.

Cai, R., Klamczynska, B., & Baik, B. K. (2001). Preparation of Bean Curds from Protein Fractions of Six Legumes. *Journal of Agricultural and Food Chemistry*, *49*(6), 3068–3073.

Carbonaro, M., Maselli, P., & Nucara, A. (2012). Relationship between digestibility and secondary

structure of raw and thermally treated legume proteins: A Fourier transform infrared (FT-IR) spectroscopic study. *Amino Acids*, *43*(2), 911–921.

Carbonaro, M., & Nucara, A. (2010). Secondary structure of food proteins by Fourier transform spectroscopy in the mid-infrared region. *Amino Acids*, *38*(3), 679–690.

Carullo, D., Abera, B. D., Casazza, A. A., Donsì, F., Perego, P., Ferrari, G., & Pataro, G. (2018). Effect of pulsed electric fields and high pressure homogenization on the aqueous extraction of intracellular compounds from the microalgae *Chlorella vulgaris*. *Algal Research, 31*, 60-69.

Cauvain, S., & Young, L. (2007). *Technology of breadmaking*. Springer New York, NY.

Çelik, M., Güzel, M., & Yildirim, M. (2019). Effect of pH on protein extraction from sour cherry kernels and functional properties of resulting protein concentrate. *Journal of Food Science and Technology, 56*(6), 3023-3032.

Chan, C.-H., Yusoff, R., Ngoh, G.-C., & Kung, F. W.-L. (2011). Microwave-assisted extractions of active ingredients from plants. *Journal of Chromatography A, 1218*(37), 6213.

Chang, L., Lan, Y., Bandillo, N., Ohm, J. B., Chen, B., & Rao, J. (2022). Plant proteins from green pea and chickpea: Extraction, fractionation, structural characterization and functional properties. *Food Hydrocolloids*, *123*, 107165.

Chemat, F., Abert Vian, M., Fabiano-Tixier, A. S., Nutrizio, M., Režek Jambrak, A., Munekata, P. E. S., Lorenzo, J. M., Barba, F. J., Binello, A., & Cravotto, G. (2020). A review of sustainable and intensified techniques for extraction of food and natural products. *Green*

Chemistry, *22*(8), 2325–2353.

Chemat, F., Abert Vian, M., Ravi, H. K., Khadhraoui, B., Hilali, S., Perino, S., & Tixier, A. F. (2019). Review of alternative solvents for green extraction of food and natural products: Panorama, principles, applications, and prospects. *Molecules, 24*(16), 3007.

Chemat, F., Rombaut, N., Sicaire, A. G., Meullemiestre, A., Fabiano-Tixier, A. S., & Abert-Vian, M. (2017). Ultrasound assisted extraction of food and natural products. Mechanisms, techniques, combinations, protocols and applications. A review. *Ultrasonics Sonochemistry*, *34*, 540–560.

Chen, B., Guo, J., Xie, Y., Zhou, K., Li, P., & Xu, B. (2021). Modulating the aggregation of myofibrillar protein to alleviate the textural deterioration of protein gels at high temperature: The effect of hydrophobic interactions. *Food Chemistry*, *341*, 128274.

Chen, F., Su, X., Gao, J., Liu, Y., Zhang, Q., & Luo, D. (2023). A modified strategy to improve the dissolution of flavonoids from Artemisiae Argyi Folium using ultrasonic-assisted enzyme-deep eutectic solvent system. *Journal of Chromatography*, *1707*, 464282.

Chen, J., Li, Y., Wang, X., & Liu, W. (2019). Application of deep eutectic solvents in food analysis: A review. *Molecules, 24*(24), 4594.

Chen, J., Ni, Y., Zhang, P., Liang, X., & Fang, S. (2023). Acidic natural deep eutectic solvents as dual solvents and catalysts for the solubilization and deglycosylation of soybean isoflavone extracts: Genistin as a model compound. *Food Chemistry*, *406*, 134999.

Chen, N., Zhao, M., Niepceron, F., Nicolai, T., & Chassenieux, C. (2017). The effect of the pH on

thermal aggregation and gelation of soy proteins. *Food Hydrocolloids*, *66*, 27–36.

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*, *9*(30), 10083–10092.

Chen, R., Meng, F., Zhang, S., & Liu, Z. (2009). Effects of ultrahigh pressure extraction conditions on yields and antioxidant activity of ginsenoside from ginseng. *Separation and Purification Technology, 66*(2), 340-346.

Chen, Y., & Mu, T. (2019). Application of deep eutectic solvents in biomass pretreatment and conversion. *Green Energy & Environment*, *4*(2), 95–115.

Chew, K. W., Chia, S. R., Lee, S. Y., Zhu, L., & Show, P. L. (2019). Enhanced microalgal protein extraction and purification using sustainable microwave-assisted multiphase partitioning technique. *Chemical Engineering Journal, 367*, 1-8.

Chia, S. R., Tang, M. S. Y., Chow, Y. H., Ooi, C. W., Rambabu, K., Zhu, L., & Show, P. L. (2019). Recent developments of reverse micellar techniques for lysozyme, bovine serum albumin, and bromelain extraction. *Molecular biotechnology, 61*(10), 715-724.

Chove, B. E., Grandison, A. S., & Lewis, M. J. (2007). Some functional properties of fractionated soy protein isolates obtained by microfiltration. *Food Hydrocolloids*, *21*, 1379–1388.

Chronakis, I. S., & Kasapis, S. (1993). Structural properties of single and mixed milk/soya protein systems. *Food Hydrocolloids*, *7*(6), 459–478.

Chronakis, I. S., Kasapis, S., Richardson, R. K., & Doxastakis, G. (1995). Characterisation of A

Commercial Soy Isolate by Physical Techniques. *Journal of Texture Studies*, *26*(4), 371–389.

Chunyan Ma, Aatto Laaksonen, Chang Liu, Xiaohua Lu, & Xiaoyan Ji. (2018). The peculiar effect of water on ionic liquids and deep eutectic solvents. *Chemical Society Reviews*, *47*(23), 8685–8720.

Clark, D. T., Peeling, J., & Colling, L. (1976). An experimental and theoretical investigation of the core level spectra of a series of amino acids, dipeptides and polypeptides. *Biochimica et Biophysica Acta*, *453*(2), 533–545. https://doi.org/10.1016/0005-2795(76)90149-5

Cui, Q., Liu, J.-Z., Wang, L.-T., Kang, Y.-F., Meng, Y., Jiao, J., & Fu, Y.-J. (2018). Sustainable deep eutectic solvents preparation and their efficiency in extraction and enrichment of main bioactive flavonoids from sea buckthorn leaves. *Journal of Cleaner Production, 184*, 826-835.

Cui, Q., Ni, X., Zeng, L., Tu, Z., Li, J., Sun, K., Chen, X., & Li, X. (2017). Optimization of protein extraction and decoloration conditions for tea residues. *Horticultural Plant Journal, 3*(4), 172-176.

Cui, Q., Wang, L., Wang, G., Zhang, A., Wang, X., & Jiang, L. (2021). Ultrasonication effects on physicochemical and emulsifying properties of Cyperus esculentus seed (tiger nut) proteins. *LWT*, *142*, 110979.

Dabbour, M., He, R., Ma, H., & Musa, A. (2018). Optimization of ultrasound assisted extraction of protein from sunflower meal and its physicochemical and functional properties. *Journal of Food Process Engineering*, *41*(5), e12799.

Daher, D., Le Gourrierec, S., & Pérez-Lamela, C. (2017). Effect of high pressure processing on the microbial inactivation in fruit preparations and other vegetable based beverages. *Agriculture and Agricultural Science Procedia, 7*(9), 72.

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*, *187*, 14–19.

de Figueiredo, V. R. G., Yamashita, F., Vanzela, A. L. L., Ida, E. I., & Kurozawa, L. E. (2018). Action of multi-enzyme complex on protein extraction to obtain a protein concentrate from okara. *Journal of Food Science and Technology, 55*(4), 1508-1517.

de Paiva Gouvêa, L., Caldeira, R., de Lima Azevedo, T., Galdeano, M. C., Felberg, I., Lima, J. R., & Grassi Mellinger, C. (2023). Physical and techno-functional properties of a common bean protein concentrate compared to commercial legume ingredients for the plant-based market. *Food Hydrocolloids*, *137*, 108351.

del Mar Contreras-Gámez, M., Galán-Martín, Á., Seixas, N., da Costa Lopes, A. M., Silvestre, A., & Castro, E. (2023). Deep eutectic solvents for improved biomass pretreatment: Current status and future prospective towards sustainable processes. *Bioresource Technology*, *369*, 128396.

Deng, G., Rodríguez-Espinosa, M. E., Yan, M., Lei, Y., Guevara-Oquendo, V. H., Feng, X., Zhang, H., Deng, H., Zhang, W., Samadi, & Yu, P. (2020). Using advanced vibrational molecular spectroscopy (ATR-Ft/IRS and synchrotron SR-IMS) to study an interaction between protein molecular structure from biodegradation residues and nutritional properties of cool-climate adapted faba bean seeds. *Molecular and Biomolecular Spectroscopy*, *229*,

117935.

Dent, T., & Maleky, F. (2023). Pulse protein processing: The effect of processing choices and enzymatic hydrolysis on ingredient functionality. *Critical Reviews in Food Science and Nutrition*, *63*(29), 9914–9925.

Dhull, S. B., Kidwai, M. K., Noor, R., Chawla, P., & Rose, P. K. (2022). A review of nutritional profile and processing of faba bean (Vicia faba L.). *Legume Science*, *4*(3), e129.

Ding, L., Lu, L., Sheng, L., Tang, C., Chen, Y., & Cai, Z. (2020). Mechanism of enhancing foaming properties of egg white by super critical carbon dioxide treatment. *Food Chemistry*, *317*, 126349.

Dombrowski, J., Gschwendtner, M., Saalfeld, D., & Kulozik, U. (2018). Salt-dependent interaction behavior of β-Lactoglobulin molecules in relation to their surface and foaming properties. *Colloids & Surface A: physiochemical and Engineering Aspects, 558,* 455-462.

Dombrowski, K. E., Wright, S. E., Birkbeck, J. C., & Moddeman, W. E. (1995). Methods in Food Structure Analysis. In M.Z. Atassi, & E. Appella (Eds.), *X-Ray Photoelectron Spectroscopy of Amino Acids, Polypeptides, and Simple Carbohydrates* (251–260). Springer Nature Link.

Dong, X., Zhao, M., Shi, J., Yang, B., Li, J., Luo, D., Jiang, G., & Jiang, Y. (2011a). Effects of combined high-pressure homogenization and enzymatic treatment on extraction yield, hydrolysis and function properties of peanut proteins. *Innovative Food Science and Emerging Technologies, 12*(4), 478-483.

Dong, X. Y., Guo, L. L., Wei, F., Li, J. F., Jiang, M. L., Li, G. M., Zhao, Y. D., & Chen, H. (2011b). Some characteristics and functional properties of rapeseed protein prepared by ultrasonication, ultrafiltration and isoelectric precipitation. *Journal of the Science of Food and Agriculture, 91*(8), 1488-1498.

Drulyte, D., & Orlien, V. (2019). The Effect of Processing on Digestion of Legume Proteins. *Foods*, *8*(6), 224.

Du, L., Arauzo, P. J., Meza Zavala, M. F., Cao, Z., Olszewski, M. P., & Kruse, A. (2020a). Towards the properties of different biomass-derived proteins via various extraction methods. *Molecules, 25*(3), 488.

Du, Y., Zhang, Q., Zhao, X., & Chen, F. (2020b). Effect of reverse micelle on physicochemical properties of soybean 7S globulins. *Journal of Food Engineering, 282*, 110026.

Eckert, E., Han, J., Swallow, K., Tian, Z., Jarpa-Parra, M., & Chen, L. (2019). Effects of enzymatic hydrolysis and ultrafiltration on physicochemical and functional properties of faba bean protein. *Cereal Chemistry*, *96*(4), 725–741.

Ekezie, F.-G. C., Sun, D.-W., & Cheng, J.-H. (2017). Acceleration of microwave-assisted extraction processes of food components by integrating technologies and applying emerging solvents: A review of latest developments. *Trends in Food Science and Technology, 67*, 160-172.

El Achkar, T., Greige-Gerges, H., & Fourmentin, S. (2021). Basics and properties of deep eutectic solvents: a review. *Environmental Chemistry Letters*, *19*(4), 3397–3408.

Elbourne, A., Besford, Q. A., Meftahi, N., Crawford, R. J., Daeneke, T., Greaves, T. L., McConville, C. F., Bryant, G., Bryant, S. J., & Christofferson, A. J. (2021). The Impact of Water on the Lateral Nanostructure of a Deep Eutectic Solvent–Solid Interface. *Australian Journal of Chemistry*, *75*(2), 111–125.

Elderderi, S., Leman-Loubière, C., Wils, L., Henry, S., Bertrand, D., Byrne, H. J., Chourpa, I., Enguehard-Gueiffier, C., Munnier, E., Elbashir, A. A., Boudesocque-Delaye, L., & Bonnier, F. (2020). ATR-IR spectroscopy for rapid quantification of water content in deep eutectic solvents. *Journal of Molecular Liquids*, *311*, 113361.

Elhag, H. E. A., Naila, A., Ajit, A., Sulaiman, A. Z., Ishak, W. F. W., Aziz, B. A., & Prasad, R. (2019). Optimization of protein yields in water extracts of *Jackiopsis ornata* roots by response surface methodology using microwave assisted extraction (MAE). *AIP Conference Proceedings* 2068 (pp. 020055): AIP Publishing LLC.

Ellman, G. L. (1959). Tissue Sulfhydryl Groups. *Archives of Biochemistry and Biophysics*, *82*, 70–77.

Elsohaimy, S. A., Refaay, T. M., & Zaytoun, M. A. M. (2015). Physicochemical and functional properties of quinoa protein isolate. *Annals of Agricultural Sciences, 60*(2), 297-305.

Esquembre, R., Sanz, J. M., Wall, J. G., Del Monte, F., Mateo, C. R., & Ferrer, M. L. (2013). Thermal unfolding and refolding of lysozyme in deep eutectic solvents and their aqueous dilutions. *Physical Chemistry Chemical Physics*, *15*(27), 11248–11256.

Faba Beans Global Market Report *(*2021). Featuring Prairie Fava, Unigrain, Roland Beans, Aviip Group, Alberta Pulse Growers, Stamp Seeds, Riddell Seed, and Pawnee Buttes Seed. -

https://www.businesswire.com/news/home/20211213005626/en/Faba-Beans-Global-Market-Report-2021.

Fadimu, G. J., Le, T. T., Gill, H., Farahnaky, A., Olatunde, O. O., & Truong, T. (2022). Enhancing the biological activities of food protein-derived peptides using non-thermal technologies: A review. *Foods, 11*(13), 1823.

FAO/WHO. (1991). Protein quality evaluation: Report of the joint FAO/WHO expert consultation. In *FAO Food and Nutrition Paper 51*.

Fayaz, G., Plazzotta, S., Calligaris, S., Manzocco, L., & Nicoli, M. C. (2019). Impact of high pressure homogenization on physical properties, extraction yield and biopolymer structure of soybean okara. *LWT, 113*, 108324.

Feng, L., Jia, X., Yan, J., Yan, W., & Yin, L. (2021). Mechanical, thermal stability and microstructural properties of emulsion-filled gels: Effect of sugar beet pectin/soy protein isolate ratio. *LWT, 141,*110917.

Feng, Z., Morton, J. D., Maes, E., Kumar, L., & Serventi, L. (2025). Proximate composition, peptide characterization and bioactive properties of faba bean blanching water. *Food Research International*, *200*, 115426.

Ferreira, A. S. D., Craveiro, R., Duarte, A. R., Barreiros, S., Cabrita, E. J., & Paiva, A. (2021). Effect of water on the structure and dynamics of choline chloride/glycerol eutectic systems. *Journal of Molecular Liquids*, *342*, 117463.

Flint, E. B., & Suslick, K. S. (1991). The temperature of cavitation. *Science, 253*(5026), 1397-1399.

Flores-Jiménez, N. T., Ulloa, J. A., Silvas, J. E. U., Ramírez, J. C. R., Ulloa, P. R., Rosales, P. U. B., Carrillo, Y. S., & Leyva, R. G. (2019). Effect of high-intensity ultrasound on the compositional, physicochemical, biochemical, functional and structural properties of canola (Brassica napus L.) protein isolate. *Food Research International*, *121*, 947–956.

Joye, I. (2019). Protein digestibility of cereal products. *Foods, 8*(6), 199.

Fu, H., Li, J., Yang, X., Swallah, M. S., Gong, H., Ji, L., Meng, X., Lyu, B., & Yu, H. (2023). The heated-induced gelation of soy protein isolate at subunit level: Exploring the impacts of α and α′ subunits on SPI gelation based on natural hybrid breeding varieties. *Food Hydrocolloids*, *134*, 108008.

Fuhrmeister, H., & Meuser, F. (2003). Impact of processing on functional properties of protein products from wrinkled peas. *Journal of Food Engineering, 56*(2-3),119-129.

Gabriele, F., Chiarini, M., Germani, R., Tiecco, M., & Spreti, N. (2019). Effect of water addition on choline chloride/glycol deep eutectic solvents: Characterization of their structural and physicochemical properties. *Journal of Molecular Liquids*, *291*, 111301.

Gadalkar, S. M., & Rathod, V. K. (2020). Extraction of watermelon seed proteins with enhanced functional properties using ultrasound. *Preparative Biochemistry and Biotechnology, 50*(2), 133-140.

Gajardo-Parra, N., Lubben, M., Winnert J.M., Leiva, A., Brennecke, J.F., and Cannales, R. I.

(2019). Physicochemical properties of choline chloride-based deep eutectic solvents and excess properties of their pseudo-binary mixtures with 1-butanol. *The Journal of Chemical Thermodynamics*, *133*, 272-284.

Gangadharappa, B. S., Dammalli, M., Rajashekarappa, S., Pandurangappa, K. M. T., & Siddaiah, G. B. (2017). Reverse micelles as a bioseparation tool for enzymes. *Proteins Proteomics, 8*(2), 105-120.

Gao, Z., Shen, P., Lan, Y., Cui, L., Ohm, J.-B., Chen, B., & Rao, J. (2020). Effect of alkaline extraction pH on structure properties, solubility, and beany flavor of yellow pea protein isolate. *Food Research International, 131*, 109045.

Gatehouse, J., Croy, R., McIntosh, R., & Paul, C. (1980). Quantitative and qualitative variation in the storage proteins of material from the EEC joint field bean test. Proceeding of the Proceedings of a seminar in the EEC Programme of Coordination of Research on the Improvement of the Production of Plant Proteins, UK, *3*, 173-190.

Ge, J., Sun, C., Chang, Y., Li, S., Zhang, Y., & Fang, Y. (2023). Understanding the differences in heat-induced gel properties of twelve legume proteins: A comparative study. *Food Research International*, *163*, 112134.

Gençdağ, E., Görgüç, A., & Yılmaz, F. M. (2021). Recent Advances in the Recovery Techniques of Plant-Based Proteins from Agro-Industrial By-Products. *Food Reviews International*, *37*(4), 447–468.

George, J., & Rastogi, N. (2017). Impact of high pressure on the infusion of curcuminoids in pineapple slices. *Journal of Food Science & Nutrition, 3*, 027.

Gereniu, C. R. N., Saravana, P. S., Getachew, A. T., & Chun, B.-S. (2017). Characteristics of functional materials recovered from Solomon Islands red seaweed (*Kappaphycus alvarezii*) using pressurized hot water extraction. *Journal of Applied Phycology, 29*(3), 1609-1621.

Gerzhova, A., Mondor, M., Benali, M., & Aider, M. (2016). Study of total dry matter and protein extraction from canola meal as affected by the pH, salt addition and use of zeta-potential/turbidimetry analysis to optimize the extraction conditions. *Food Chemistry, 201*, 243-252.

Ghisaidoobe, A. B. T., & Chung, S. J. (2014). Intrinsic Tryptophan Fluorescence in the Detection and Analysis of Proteins: A Focus on Förster Resonance Energy Transfer Techniques. *International Journal of Molecular Sciences*, *15*(12), 22518–22538.

Gochev, G., Retzlaff, I., Exerowa, D., Miller, R. (2014). Electrostatic stabilization of foam films from β-lactoglobulin solutions. *Colloids & Surface A: physiochemical and Engineering Aspects, 460,* 272-279.

González-Pérez, S., & Vereijken, J. M. (2007). Sunflower proteins: Overview of their physicochemical, structural and functional properties. *Journal of the Science of Food and Agriculture, 87*(12), 2173-2191.

Görgüç, A., Bircan, C., & Yılmaz, F. M. (2019). Sesame bran as an unexploited by-product: Effect of enzyme and ultrasound-assisted extraction on the recovery of protein and antioxidant compounds. *Food Chemistry*, *283*, 637–645.

Görgüç, A., Özer, P., & Yılmaz, F. M. (2020). Microwave-assisted enzymatic extraction of plant protein with antioxidant compounds from the food waste sesame bran: Comparative optimization study and identification of metabolomics using LC/Q-TOF/MS. *Journal of Food Processing and Preservation, 44*(1), e14304.

Grandview Research. (2021). Protein Ingredients Market Size, Share & Trends Analysis Report By Product (Plant Proteins, Animal/Dairy Proteins, Microbe-based Proteins, Insect Proteins), By Application, By Region, And Segment Forecasts, 2021 - 2028. https://www.grandviewresearch.com/industry-analysis/protein-ingredients-market

Grossmann, L., & McClements, D. J. (2023). Current insights into protein solubility: A review of its importance for alternative proteins. *Food Hydrocolloids*, *137*, 108416.

Grudniewska, A., Melo, E. M. de, 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*, *6*(11), 15791–15800.

Guerrero, P., Garrido, T., Leceta, I., & De La Caba, K. (2013). Films based on proteins and polysaccharides: Preparation and physical–chemical characterization. *European Polymer Journal*, *49*(11), 3713–3721.

Gulzar, S., Martín-Belloso, O., & Soliva-Fortuny, R. (2024). Tailoring the Techno-Functional Properties of Fava Bean Protein Isolates: A Comparative Evaluation of Ultrasonication and Pulsed Electric Field Treatments. *Foods, 13*(3), 376.

Gundogan, R., & Karaca, A.C. (2020). Physicochemical and functional properties of proteins

isolated from local beans of Turkey. *LWT, 130, 109609.*

Gunny, A. A. N., Arbain, D., Nashef, E. M., & Jamal, P. (2015). Applicability evaluation of Deep Eutectic Solvents–Cellulase system for lignocellulose hydrolysis. *Bioresource Technology*, *181*, 297–302.

Guo, Z., Chen, F., Yang, H., Liu, K., & Zhang, L. (2015). Kinetics of protein extraction in reverse micelle. *International Journal of Food Properties, 18*(8), 1707-1718.

Guzmán-Lorite, M., Marina, M. L., & García, M. C. (2022). Pressurized liquids vs. high intensity focused ultrasounds for the extraction of proteins from a pomegranate seed waste. *Innovative Food Science and Emerging Technologies, 77*, 102958.

Hadidi, M., Khaksar, F. B., Pagan, J., & Ibarz, A. (2020). Application of ultrasound-ultrafiltration-assisted alkaline isoelectric precipitation (UUAAIP) technique for producing alfalfa protein isolate for human consumption: Optimization, comparison, physicochemical, and functional properties. *Food Research International, 130*, 108907.

Hammond, O. S., Bowron, D. T., Edler, K. J., Hammond, S., Edler, K. J., & B owron, D. T. (2017). The Effect of Water upon Deep Eutectic Solvent Nanostructure: An Unusual Transition from Ionic Mixture to Aqueous Solution. *Angewandte Chemie*, *129*(33), 9914–9917.

Hansen, B. B., Spittle, S., Chen, B., Poe, D., Zhang, Y., Klein, J. M., Horton, A., Adhikari, L., Zelovich, T., Doherty, B. W., Gurkan, B., Maginn, E. J., Ragauskas, A., Dadmun, M., Zawodzinski, T. A., Baker, G. A., Tuckerman, M. E., Savinell, R. F., & Sangoro, J. R. (2021a). Deep Eutectic Solvents: A Review of Fundamentals and Applications. *Chemical Reviews*, *121*(3), 1232–1285.

Hansen, B. B., Spittle, S., Chen, B., Poe, D., Zhang, Y., Klein, J. M., Horton, A., Adhikari, L., Zelovich, T., Doherty, B. W., Gurkan, B., Maginn, E. J., Ragauskas, A., Dadmun, M., Zawodzinski, T. A., Baker, G. A., Tuckerman, M. E., Savinell, R. F., & Sangoro, J. R. (2021b). Deep Eutectic Solvents: A Review of Fundamentals and Applications. *Chemical Reviews*, *121*(3), 1232–1285.

Haris, P. I., & Severcan, F. (1999). FTIR spectroscopic characterization of protein structure in aqueous and non-aqueous media. *Journal of Molecular Catalysis B: Enzymatic*, *7*(1–4), 207–221.

Hawthorne, S. B., Grabanski, C. B., Martin, E., & Miller, D. J. (2000). Comparisons of Soxhlet extraction, pressurized liquid extraction, supercritical fluid extraction and subcritical water extraction for environmental solids: recovery, selectivity and effects on sample matrix. *Journal of Chromatography A, 892*(1-2), 421-433.

He, Z., Li, W., Guo, F., Li, W., Zeng, M., & Chen, J. (2015). Foaming Characteristics of Commercial Soy Protein Isolate as Influenced by Heat-Induced Aggregation. *International Journal of Food Properties*, *18*(8), 1817–1828.

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*, *60*, 102314.

Hernández-Marín, C. R., Guadarrama-Mendoza, P. C., Valadez-Blanco, R., Chen, B., & Diosady, L. L. (2019). Alkaline extraction and purification of huauzontle (*Chenopodium berlandieri*

subsp. Nuttalliae) seed proteins by ultrafiltration membranes. *IEEE International Conference on Applied Science and Advanced Technology (iCASAT)*, (pp. 1-6).

Hertzler, S. R., Lieblein-Boff, J. C., Weiler, M., & Allgeier, C. (2020). Plant Proteins: Assessing Their Nutritional Quality and Effects on Health and Physical Function. *Nutrients*, *12*(12), 3704.

Hewage, A., Olatunde, O., Nimalaratne, C., House, J. D., Aluko, R. E., & Bandara, N. (2024). Improved protein extraction technology using deep eutectic solvent system for producing high purity fava bean protein isolates at mild conditions. *Food Hydrocolloids*, *147*, 109283.

Hewage, A., Olatunde, O. O., Nimalaratne, C., Malalgoda, M., Aluko, R. E., & Bandara, N. (2022). Novel Extraction technologies for developing plant protein ingredients with improved functionality. *Trends in Food Science & Technology*, *129*, 492–511.

Hou, F., Ding, W., Qu, W., Oladejo, A. O., Xiong, F., Zhang, W., He, R., & Ma, H. (2017). Alkali solution extraction of rice residue protein isolates: Influence of alkali concentration on protein functional, structural properties and lysinoalanine formation. *Food Chemistry*, *218*, 207–215.

Hou, W., Zhang, X., Yan, Q., Li, P., Sha, W., Tian, Y., & Liu, Y. (2018). Linkage Map of a Gene Controlling Zero Tannins (zt-1) in Faba Bean (Vicia faba L.) with SSR and ISSR Markers. *Agronomy, 8*(6), 80.

Hsu, H. W., Vavak, D. L., Satterlee, L. D., & Mmiller, G. A. (1977). A multienzime technique for estimating protein digestibility. *Journal of Food Science*, *42*, 1269–1273.

Hu, H., Cheung, I. W. Y., Pan, S., & Li-Chan, E. C. Y. (2015). Effect of high intensity ultrasound

on physicochemical and functional properties of aggregated soybean β-conglycinin and glycinin. *Food Hydrocolloids*, *45*, 102–110.

Hu, H., Wu, J., Li-Chan, E. C. Y., Zhu, L., Zhang, F., Xu, X., Fan, G., Wang, L., Huang, X., & Pan, S. (2013). Effects of ultrasound on structural and physical properties of soy protein isolate (SPI) dispersions. *Food Hydrocolloids*, *30*(2), 647–655.

Hua, Y., Cui, S. W., Wang, Q., Mine, Y., & Poysa, V. (2005). Heat induced gelling properties of soy protein isolates prepared from different defatted soybean flours. *Food Research International*, *38*(4), 377–385.

Husband, F. A., Wilde, P. J., Clark, D. C., Rawel, H. M., & Muschiolik, G. (1994). Foaming properties of modified faba bean protein isolates. *Topics in Catalysis*, *8*(5), 455–468.

Hunter, M. C., Smith, R. G., Schipanski, M. E., Atwood, L. W., & Mortensen, D. A. (2017). Agriculture in 2050: Recalibrating targets for sustainable intensification. *BioScience, 67*(4), 386-391.

Iqbal, M., Tao, Y., Xie, S., Zhu, Y., Chen, D., Wang, X., Huang, L., Peng, D., Sattar, A., Shabbir, M. A. B., Hussain, H. I., Ahmed, S., & Yuan, Z. (2016). Aqueous two-phase system (ATPS): An overview and advances in its applications. *Biological Procedures Online, 18*(1), 18.

Ismail, B. P., Senaratne-Lenagala, L., Stube, A., & Brackenridge, A. (2020). Protein demand: review of plant and animal proteins used in alternative protein product development and production. *Animal Frontiers, 10*(4), 53-63.

Jaeschke, D. P., Mercali, G. D., Marczak, L. D. F., Müller, G., Frey, W., & Gusbeth, C. (2019). Extraction of valuable compounds from *Arthrospira platensis* using pulsed electric field treatment. *Bioresource Technology, 283*, 207-212.

Jain, A., Prakash, M., & Radha, C. (2015). Extraction and evaluation of functional properties of groundnut protein concentrate. *Journal of Food Science and Technology, 52*(10), 6655-6662.

Janssen, S. W. P. M., Pouvreau, L., & de Vries, R. J. (2024). Commercial plant protein isolates: The effect of insoluble particles on gelation properties. *Food Hydrocolloids*, *154*, 110049.

Jeganathan, B., Vasanthan, T., & Temelli, F. (2023). Isolation of clean-label faba bean (Vicia faba L.) proteins: A comparative study of mild fractionation methods against traditional technologies. *Innovative Food Science & Emerging Technologies*, *84*, 103285.

Jiang, J., Chen, J., & Xiong, Y. L. (2009). Structural and Emulsifying Properties of Soy Protein Isolate Subjected to Acid and Alkaline pH-Shifting Processes. *Journal of Agricultural and Food Chemistry*, *57*(16), 7576–7583.

Jiang, L., Wang, J., Li, Y., Wang, Z., Liang, J., Wang, R., Chen, Y., Ma, W., Qi, B., & Zhang, M. (2014). Effects of ultrasound on the structure and physical properties of black bean protein isolates. *Food Research International*, *62*, 595–601.

Jiang, L., Wang, Z., Li, Y., Meng, X., Sui, X., Qi, B., & Zhou, L. (2015). Relationship Between Surface Hydrophobicity and Structure of Soy Protein Isolate Subjected to Different Ionic Strength. *International Journal of Food Properties*, *18*(5), 1059–1074.

Jiang, X., Gao, F., Ma, Y., Huo, N., Guo, Y., & Yu, Y. (2024). Protein from tiger nut meal extracted by deep eutectic solvent and alkali-soluble acid precipitation: A comparative study on structure, function, and nutrition. *Food Chemistry*, *452*, 139608.

Jiang, Y., Zhou, X., Zheng, Y., Wang, D., Deng, Y., & Zhao, Y. (2021). Impact of ultrasonication/shear emulsifying/microwave-assisted enzymatic extraction on rheological, structural, and functional properties of *Akebia trifoliata* (Thunb.) Koidz. seed protein isolates. *Food Hydrocolloids, 112*, 106355.

Joehnke, M. S., Jeske, S., Ispiryan, L., Zannini, E., Arendt, E. K., Bez, J., Sørensen, J. C., & Petersen, I. L. (2021). Nutritional and anti-nutritional properties of lentil (Lens culinaris) protein isolates prepared by pilot-scale processing. *Food Chemistry: X*, *9*, 100112.

Johansson, M., Karkehabadi, S., Johansson, D. P., & Langton, M. (2023). Gelation behaviour and gel properties of the 7S and 11S globulin protein fractions from faba bean (Vicia faba var. minor) at different NaCl concentrations. *Food Hydrocolloids*, *142*, 108789.

John, H., & Mansuri, S. M. (2018). Rheological Properties and Particle Size Distribution of Soy Protein Isolate as Affected by Drying Methods. *International Journal of Nutrition & Food Sciecnes, 7*(5), 555721.

John, H., & Sinha, L. (2019). Quality characteristics and functional properties of soy protein isolate prepared using ultrafiltration. *Journal of Agricultural Engineering, 56*(1), 16-28.

Ju, Q., Yuan, Y., Wu, C., Hu, Y., Zhou, S., & Luan, G. (2023). Heat-induced aggregation of subunits/polypeptides of soybean protein: Structural and physicochemical properties. *Food Chemistry*, *405*, 134774.

Ju, Z., & Howard, L. R. (2005). Subcritical water and sulfured water extraction of anthocyanins and other phenolics from dried red grape skin. *Journal of Food Science, 70*(4), S270-S276.

Jung, S., Lamsal, B. P., Stepien, V., Johnson, L. A., & Murphy, P. A. (2006). Functionality of soy protein produced by enzyme-assisted extraction. *Journal of the American Oil Chemists' Society, 83*(1), 71-78.

Kadam, S. U., Tiwari, B. K., & O'Donnell, C. P. (2013). Application of novel extraction technologies for bioactives from marine algae. *Journal of Agricultural and Food Chemistry, 61*(20), 4667-4675.

Kalaydzhiev, H., Georgiev, R., Ivanova, P., Stoyanova, M., Silva, C. L. M., & Chalova, V. I. (2020). Enhanced solubility of rapeseed meal protein isolates prepared by sequential isoelectric precipitation. *Foods, 9*(6), 703.

Kalla-Bertholdt, A. M., Baier, A. K., & Rauh, C. (2023). Influence of High-Intensity Ultrasound on Characteristics and Bioaccessibility of Pea Protein in Fiber-Enriched Suspensions. *Foods*, *12*(17), 3160.

Kamani, M. H., Liu, J., Fitzsimons, S. M., Fenelon, M. A., & Murphy, E. G. (2024). Determining the influence of fava bean pre-processing on extractability and functional quality of protein isolates. *Food Chemistry: X*, *21*, 101200.

Kapoore, R. V., Butler, T. O., Pandhal, J., & Vaidyanathan, S. (2018). Microwave-assisted extraction for microalgae: From biofuels to biorefinery. *Biology, 7*(1), 18.

Karaca, A. C., Low, N., & Nickerson, M. (2011a). Emulsifying properties of canola and flaxseed protein isolates produced by isoelectric precipitation and salt extraction. *Food Research International, 44*(9), 2991-2998.

Karaca, A. C., Low, N., & Nickerson, M. (2011). Emulsifying properties of chickpea, faba bean, lentil and pea proteins produced by isoelectric precipitation and salt extraction. *Food Research International*, *44*(9), 2742–2750.

Kareem, M. A., Mjalli, F. S., Hashim, M. A., & Alnashef, I. M. (2010). *Phosphonium-Based Ionic Liquids Analogues and Their Physical Properties*. *ACS susutainable Chemistry and Engineering, 7* (3), 2933-2942.

Karimi, A., Bhowmik, P., Yang, T. C., Samaranayaka, A., & Chen, L. (2024). Extraction of canola protein via natural deep eutectic solvents compared to alkaline treatments: Isolate characteristics and protein structural and functional properties. *Food Hydrocolloids*, *152*, 109922.

Karkanis, A., Ntatsi, G., Lepse, L., Fernández, J. A., Vågen, I. M., Rewald, B., Alsiņa, I., Kronberga, A., Balliu, A., Olle, M., Bodner, G., Dubova, L., Rosa, E., & Savvas, D. (2018). Faba bean cultivation – revealing novel managing practices for more sustainable and competitive European cropping systems. *Frontiers in Plant Science*, *9*, 375969.

Kasai, N., & Ikehara, H. (2005). Stepwise Extraction of Proteins and Carbohydrates from Soybean Seed. *Journal of Agricultural and Food Chemistry*, *53*(10), 4245–4252.

Kato, A., & Nakai, S. (1980). Hydrophobicity Determined by A Fluorescence Probe Method and its Correlation with Surface Properties of Proteins. *Biochimica et Biophysica Acta*, *624*, 13–

20.

Kaur, M., & Singh, N. (2007). Characterization of protein isolates from different Indian chickpea (Cicer arietinum L.) cultivars. *Food Chemistry*, *102*(1), 366–374.

Kaur, S., Kumari, M., & Kashyap, H. K. (2020). Microstructure of Deep Eutectic Solvents: Current Understanding and Challenges. *Journal of Physical Chemistry B*, *124*(47), 10601–10616.

Keerati-u-rai, M., Miriani, M., Iametti, S., Bonomi, F., & Corredig, M. (2012). Structural changes of soy proteins at the oil–water interface studied by fluorescence spectroscopy. *Colloids and Surfaces B: Biointerfaces*, *93*, 41–48.

Keivaninahr, F., Gadkari, P., Benis, K. Z., Tulbek, M., & Ghosh, S. (2021). Prediction of emulsification behaviour of pea and faba bean protein concentrates and isolates from structure-functionality analysis. *RSC Advances, 11,* 12117-12135.

Khadhraoui, B., Turk, M., Fabiano-Tixier, A. S., Petitcolas, E., Robinet, P., Imbert, R., Maâtaoui, M. El, & Chemat, F. (2018). Histo-cytochemistry and scanning electron microscopy for studying spatial and temporal extraction of metabolites induced by ultrasound. Towards chain detexturation mechanism. *Ultrasonics Sonochemistry*, *42*, 482–492.

Khalil, A. H., & Mansour, E. H. (1995). The effect of cooking, autoclaving and germination on the nutritional quality of faba beans. *Food Chemistry*, *54*, 177–182.

Khan, S. A., Aslam, R., & Makroo, H. A. (2019). High pressure extraction and its application in the extraction of bio-active compounds: A review. *Journal of Food Process Engineering, 42*(1), e12896.

Khazaei, H., Purves, R. W., Hughes, J., Link, W., O'Sullivan, D. M., Schulman, A. H., Björnsdotter, E., Geu-Flores, F., Nadzieja, M., Andersen, S. U., Stougaard, J., Vandenberg, A., & Stoddard, F. L. (2019). Eliminating vicine and convicine, the main anti-nutritional factors restricting faba bean usage. *Trends in Food Science & Technology*, *91*, 549–556.

Kimura, A., Takako, F., Meili, Z., Shiori, M., Maruyama, N., & Utsumi, S. (2008). Comparison of Physicochemical Properties of 7S and 11S Globulins from Pea, Fava Bean, Cowpea, and French Bean with Those of Soybean—French Bean 7S Globulin Exhibits Excellent Properties. *Journal of Agricultural and Food Chemistry*, *56*(21), 10273–10279.

Kinsella, J. E. (1979). Functional properties of soy proteins. *Journal of the American Oil Chemists' Society*, *56*(3), 242–258. https://doi.org/10.1007/BF02671468

Klupšaitė, D., & Juodeikienė, G. (2015). Legume: Composition, protein extraction and functional properties. A review. *Chemical Technology, 66*(1), 5-12.

Kohyama, K., Sano, Y., & Doi, E. (1995). Rheological Characteristics and Gelation Mechanism of Tofu (Soybean Curd). *Journal of Agricultural and Food Chemistry*, *43*(7), 1808–1812.

Kolpakova, V. V., Lukin, N. D., & Gaivoronskaya, I. S. (2018). Global wheat production. In S. Fahad, A. Basir, & M. Adnan (Eds.), *Interrelation of functional properties of protein products from wheat with the composition and physicochemical characteristics of their proteins* (pp.205-220). IntechOpen.

Kong, J., & Yu, S. (2007). Fourier Transform Infrared Spectroscopic Analysis of Protein Secondary Structures. *Acta Biochimica et Biophysica Sinica*, *39*(8), 549–559.

Krause, M., Sørensen, J. C., Petersen, I. L., Duque-Estrada, P., Cappello, C., Tlais, A. Z. A., Di Cagno, R., Ispiryan, L., Sahin, A. W., Arendt, E. K., & Zannini, E. (2023). Associating Compositional, Nutritional and Techno-Functional Characteristics of Faba Bean (Vicia faba L.) Protein Isolates and Their Production Side-Streams with Potential Food Applications. *Foods*, *12*(5), 919.

Kudre, T. G., Benjakul, S., & Kishimura, H. (2013). Comparative study on chemical compositions and properties of protein isolates from mung bean, black bean and bambara groundnut. *Article in Journal of the Science of Food and Agriculture*, *93*(10), 2429-2436.

Kumar, M., Tomar, M., Potkule, J., Reetu., Punia, S., Dhakane-Led, J., Singh, S., Dhumal, S., Pradhan, P. C., Bhushan, B., Anitha, T., Alajil, O., Alhariri, A., Amarowicz, R., & Kennedy, J.F. (2022). Functional characterization of plant-based protein to determine its quality for food applications. *Food Hydrocolloids*, *123*, 106986.

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. (2021). Advances in the plant protein extraction: Mechanism and recommendations. In *Food Hydrocolloids, 115*, 106595.

Kumari, P., Shobhna, Kaur, S., & Kashyap, H. K. (2018). Influence of Hydration on the Structure of Reline Deep Eutectic Solvent: A Molecular Dynamics Study. *ACS Omega*, *3*(11), 15246–15255.

Kumar, S. P., Prasad, S. R., Banerjee, R., Agarwal, D. K., Kulkarni, K. S., & Ramesh, K. V. (2017). Green solvents and technologies for oil extraction from oilseeds. *Chemistry Central Journal,*

11(1), 1-7.

Laemmli, U. K. (1970). Cleavage of Structural Proteins during the Assembly of the Head of Bacteriophage T4. *Nature, 227*(5259), 680–685.

Lafarga, T., Álvarez, C., Bobo, G., & Aguiló-Aguayo, I. (2018). Characterization of functional properties of proteins from Ganxet beans (*Phaseolus vulgaris* L. var. Ganxet) isolated using an ultrasound-assisted methodology. *LWT, 98*, 106-112.

Lam, A. C. Y., Can Karaca, A., Tyler, R. T., & Nickerson, M. T. (2018). Pea protein isolates: Structure, extraction, and functionality. *Food Reviews International, 34* (2), 126–147.

Langton, M., Ehsanzamir, S., Karkehabadi, S., Feng, X., Johansson, M., & Johansson, D. P. (2020). Gelation of faba bean proteins - Effect of extraction method, pH and NaCl. *Food Hydrocolloids*, *103*, 105622.

Latif, S., & Anwar, F. (2011). Aqueous enzymatic sesame oil and protein extraction. *Food Chemistry*, *125*(2), 679–684.

Lee, H., Yildiz, G., dos Santos, L. C., Jiang, S., Andrade, J. E., Engeseth, N. J., & Feng, H. (2016). Soy protein nano-aggregates with improved functional properties prepared by sequential pH treatment and ultrasonication. *Food Hydrocolloids*, *55*, 200–209.

Li, B., Xiao, T., Guo, S., Wu, Y., Lai, R., Liu, Z., Luo, W., & Xu, Y. (2023). Oxymatrine-fatty acid deep eutectic solvents as novel penetration enhancers for transdermal drug delivery: Formation mechanism and enhancing effect. *International Journal of Pharmaceutics*, *637*, 122880.

Li, H., Yu, J., Ahmedna, M., & Goktepe, I. (2013). Reduction of major peanut allergens Ara h 1 and Ara h 2, in roasted peanuts by ultrasound assisted enzymatic treatment. *Food Chemistry, 141*(2), 762-768.

Li, J., Yang, X., Swallah, M. S., Fu, H., Ji, L., Meng, X., Yu, H., & Lyu, B. (2021). Soy protein isolate: an overview on foaming properties and air–liquid interface. *International Journal of Food Science and Technology*, *57*(1), 188–200.

Li, L., Zhou, Y., Teng, F., Zhang, S., Qi, B., Wu, C., Tian, T., Wang, Z., & Li, Y. (2020). Application of ultrasound treatment for modulating the structural, functional and rheological properties of black bean protein isolates. *International Journal of Food Science and Technology*, *55*(4), 1637–1647.

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, 152*, 23-32.

Li, P., Yang, C., Jiang, Z., Jin, Y., & Wu, W. (2023). Lignocellulose pretreatment by deep eutectic solvents and related technologies: A review. *Journal of Bioresources and Bioproducts*, *8*(1), 33–44.

Li, X., & Row, K. H. (2016). Development of deep eutectic solvents applied in extraction and separation. *Journal of Separation Science*, *39*(18), 3505–3520.

Li, Y., Chen, F., Zhang, L., & Yao, Y. (2015). Effect of surface changes of soy protein materials on water resistance. *Materials Letters*, *149*, 120–122.

Li, Y., Chen, Z., & Mo, H. (2007). Effects of pulsed electric fields on physicochemical properties of soybean protein isolates. *LWT - Food Science and Technology, 40*(7), 1167-1175.

Lima, R. R., Stephani, R., Perrone, Í. T., & de Carvalho, A. F. (2023). Plant-based proteins: A review of factors modifying the protein structure and affecting emulsifying properties. *Food Chemistry Advances*, *3*, 100397.

Lin, D., Zhang, L., Li, R., Zheng, B., Rea, M. C., & Miao, S. (2019). Effect of plant protein mixtures on the microstructure and rheological properties of myofibrillar protein gel derived from red sea bream (Pagrosomus major). *Food Hydrocolloids*, *96*, 537–545.

Lin, J., Xiang, H., Sun-Waterhouse, D., Cui, C., & Wang, W. (2022). Deep eutectic solvents and alkaline extraction of protein from seabuckthorn seed meal: a comparison study. *Food Science and Human Wellness*, *11*, 1028–1035.

Lin, Z., Jiao, G., Zhang, J., Celli, G. B., & Brooks, M. S.-L. (2020). Optimization of protein extraction from bamboo shoots and processing wastes using deep eutectic solvents in a biorefinery approach. *Biomass Conversion and Biorefinery 2020*, 1–12.

Liu, C., Pei, R., & Heinonen, M. (2021). Faba bean protein: A promising plant-based emulsifier for improving physical and oxidative stabilities of oil-in-water emulsions. *Food Chemistry, 369,* 130879.

Liu, F. F., Li, Y. Q., Wang, C. Y., Zhao, X. Z., Liang, Y., He, J. X., & Mo, H. Z. (2021). Impact of pH on the physicochemical and rheological properties of mung bean (Vigna radiata L.) protein. *Process Biochemistry*, *111*, 274–284.

Liu, J., Guan, X., Zhu, D., & Sun, J. (2008). Optimization of the enzymatic pretreatment in oat bran protein extraction by particle swarm optimization algorithms for response surface modeling. *LWT - Food Science and Technology*, *41*(10), 1913–1918.

Liu, L. H., Hung, T. V., & Bennett, L. (2008). Extraction and Characterization of Chickpea (Cicer arietinum) Albumin and Globulin. *Journal of Food Science*, *73*(5), C299–C305.

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, 10*(6), 1669-1680.

Liu, Y., Dong, X., & Sun, Y. (2008). New development of reverse micelles and applications in protein separation and refolding. *Chinese Journal of Chemical Engineering, 16*(6), 949-955.

Liu, Y., Friesen, J. B., McAlpine, J. B., Lankin, D. C., Chen, S.-N., & Pauli, G. F. (2018). Natural Deep Eutectic Solvents: Properties, Applications, and Perspectives. *Journal of Natural Products*, *81*(3), 679.

Lonnie, M., Laurie, I., Myers, M., Horgan, G., Russell, W. R., & Johnstone, A. M. (2020). Exploring health-promoting attributes of plant proteins as a functional ingredient for the food sector: A systematic review of human interventional studies. *Nutrients, 12*(8), 2291.

López, D. N., Galante, M., Robson, M., Boeris, V., & Spelzini, D. (2018). Amaranth, quinoa and chia protein isolates: Physicochemical and structural properties. *International Journal of Biological Macromolecules*, *109*, 152–159.

Lu, W., Chen, X.-W., Wang, J.-M., Yang, X.-Q., & Qi, J.-R. (2016). Enzyme-assisted subcritical water extraction and characterization of soy protein from heat-denatured meal. *Journal of Food Engineering, 169*, 250-258.

Luangthongkam, P., Fang, L., Noomhorm, A., & Lamsal, B. (2015). Addition of cellulolytic enzymes and phytase for improving ethanol fermentation performance and oil recovery in corn dry grind process. *Industrial Crops and Products, 77*, 803-808.

Luís, I. M., Alexandre, B. M., Oliveira, M. M., & Abreu, I. A. (2016). Selection of an appropriate protein extraction method to study the phosphoproteome of maize photosynthetic tissue. *PLoS One, 11*(10), e0164387.

Luo, L., Cheng, L., Zhang, R., & Yang, Z. (2022). Impact of high-pressure homogenization on physico-chemical, structural, and rheological properties of quinoa protein isolates. *Food Structure, 32*, 100265.

Luthria, D., John, K. M., *Marupaka, R., & Natarajan, S.* (2018). Recent update on methodologies for extraction and analysis of soybean seed proteins. *Journal of the Science of Food and Agriculture, 98*(15), 5572–5580.

Ma, K. K., Greis, M., Lu, J., Nolden, A. A., McClements, D. J., & Kinchla, A. J. (2022). Functional Performance of Plant Proteins. *Foods*, *11*(4), 594.

Malik, M. A., Sharma, H. K., & Saini, C. S. (2017). High intensity ultrasound treatment of protein isolate extracted from dephenolized sunflower meal: Effect on physicochemical and functional properties. *Ultrasonics Sonochemistry*, *39*, 511–519.

Malik, M. A., & Saini, C. S. (2017). Polyphenol removal from sunflower seed and kernel: Effect on functional and rheological properties of protein isolates. *Food Hydrocolloids, 63*, 705-715.

Malomo, S. A., He, R., & Aluko, R. E. (2014). Structural and Functional Properties of Hemp Seed Protein Products. *Journal of Food Science*, *79*(8), C1512–C1521.

Manasi, I., Bryant, S. J., Hammond, O. S., & Edler, K. J. (2021). Interactions of water and amphiphiles with deep eutectic solvent nanostructures. *Advances in Botanical Research*, *97*, 41–68.

Martineau-Côté, D., Achouri, A., Karboune, S., L'Hocine L. (2022) Faba bean: an untapped source of quality plant proteins and bioactives. *Nutrients, 14*(8), 1541.

Martínez-Padilla, L. P., Hernández-Rojas, F. S., Sosa-Herrera, M. G., & Juliano, P. (2022). Novel application of ultrasound and microwave-assisted methods for aqueous extraction of coconut oil and proteins. *Journal of Food Science and Technology*, *59*, 3857-3866.

Martínez-Velasco, A., Lobato-Calleros, C., Hernández-Rodríguez, B. E., Román-Guerrero, A., Alvarez-Ramirez, J., & Jaime Vernon-Carter, E. (2018). High intensity ultrasound treatment of faba bean (Vicia faba L.) protein: Effect on surface properties, foaming ability and structural changes. Ultrasonics Sonochemistry, *44,* 97-105.

Martinez, M., Stone, A. K., Yovchev, A. G., Peter, R., Vandenberg, A., & Nickerson, M. T. (2016). Effect of genotype and environment on the surface characteristics and functionality of air-classified faba bean protein concentrates. *European Food Research and Technology*, *242*(11), 1903–1911.

Mason, T. J., Chemat, F., & Ashokkumar, M. (2015). Power ultrasonics for food processing. In *Power ultrasonics*, (pp. 815-843): Elsevier.

Mayer Labba, I. C., Frøkiær, H., & Sandberg, A. S. (2021). Nutritional and antinutritional composition of fava bean (Vicia faba L., var. minor) cultivars. *Food Research International*, *140*, 110038.

Mengozzi, A., Chiavaro, E., Barbanti, D., & Bot, F. (2024). Heat-Induced Gelation of Chickpea and Faba Bean Flour Ingredients. *Gels*, *10*(5), 309.

Mession, J. L., Assifaoui, A., Cayot, P., & Saurel, R. (2012). Effect of pea proteins extraction and vicilin/legumin fractionation on the phase behavior in admixture with alginate. *Food Hydrocolloids*, *29*(2), 335–346.

Mession, J. L., Chihi, M. L., Sok, N., & Saurel, R. (2015). Effect of globular pea proteins fractionation on their heat-induced aggregation and acid cold-set gelation. *Food Hydrocolloids*, *46*, 233–243.

Mession, J. L., Sok, N., Assifaoui, A., & Saurel, R. (2013). Thermal denaturation of pea globulins (Pisum sativum L.) - Molecular interactions leading to heat-induced protein aggregation. *Journal of Agricultural and Food Chemistry*, *61*(6), 1196–1204.

Millar, K. A., Gallagher, E., Burke, R., McCarthy, S., & Barry-Ryan, C. (2019). Proximate composition and anti-nutritional factors of fava-bean (Vicia faba), green-pea and yellow-pea (Pisum sativum) flour. *Journal of Food Composition and Analysis*, *82*, 103233.

Mínguez, M. I., & Rubiales, D. (2021). Crop Physiology Case Histories for Major Crops. In V.

Sadras, & D. Calderini (Eds.), *Fava bean* (pp.452–481). Academic Press.

Mir, N. A., Riar, C. S., & Singh, S. (2019). Physicochemical, molecular and thermal properties of high-intensity ultrasound (HIUS) treated protein isolates from album (Chenopodium album) seed. *Food Hydrocolloids*, *96*, 433–441.

Mohd-Setapar, S. H., Mohamad-Aziz, S. N., Chuong, C. S., Che Yunus, M. A., Ahmad Zaini, M. A., & Kamaruddin, M. J. (2014). A review of mixed reverse micelle system for antibiotic recovery. *Chemical Engineering Communications, 201*(12), 1664-1685.

Monhemi, H., Housaindokht, M. R., Moosavi-Movahedi, A. A., & Bozorgmehr, M. R. (2014). How a protein can remain stable in a solvent with high content of urea: insights from molecular dynamics simulation of Candida antarctica lipase B in urea : choline chloride deep eutectic solvent. *Physical Chemistry Chemical Physics*, *16*(28), 14882–14893.

Monteiro, S. R., & Lopes-da-Silva, J. A. (2019). Critical evaluation of the functionality of soy protein isolates obtained from different raw materials. *European Food Research and Technology*, *245*(1), 199–212.

Moreno, H. M., Domínguez-Timón, F., Díaz, M. T., Pedrosa, M. M., Borderías, A. J., & Tovar, C. A. (2020). Evaluation of gels made with different commercial pea protein isolate: Rheological, structural and functional properties. *Food Hydrocolloids*, *99*.

Mortensen, A., & Skibsted, L.H. (2000). Kinetics and mechanism of the primary steps of degradation of carotenoids by acid in homogeneous solution. *ACS Publications*, *48*(2), 279–286.

Multari, S., Stewart, D., & Russell, W. R. (2015). Potential of Fava Bean as Future Protein Supply to Partially Replace Meat Intake in the Human Diet. *Comprehensive Reviews in Food Science and Food Safety*, *14*(5), 511–522.

Nadar, S. S., Rao, P., & Rathod, V. K. (2018). Enzyme assisted extraction of biomolecules as an approach to novel extraction technology: A review. *Food Research International, 108*, 309-330.

Nadeeshani, H., Senevirathne, N., Somaratne, G., & Bandara, N. (2021). Recent Trends in the Utilization of Pulse Protein in Food and Industrial Applications. *ACS Food Science and Technology*, *2*(5), 722-737.

Narale, B. A., Mounika, A., & Shanmugam, A. (2024). Modifications of physicochemical, functional, structural, and nutritional properties of a field bean protein isolate obtained using batch and continuous ultrasound systems. *Sustainable Food Technology*, *2*(2), 470–484.

Naseri, A., Marinho, G. S., Holdt, S. L., Bartela, J. M., & Jacobsen, C. (2020). Enzyme-assisted extraction and characterization of protein from red seaweed *Palmaria palmata*. *Algal Research, 47*, 101849.

Nasrollahzadeh, F., Roman, L., Skov, K., Jakobsen, L. M. A., Trinh, B. M., Tsochatzis, E. D., Mekonnen, T., Corredig, M., Dutcher, J. R., & Martinez, M. M. (2023). A comparative investigation of seed storage protein fractions: The synergistic impact of molecular properties and composition on anisotropic structuring. *Food Hydrocolloids*, *137*, 108400.

Nawaz, A., Irshad, S., Ali Khan, I., Khalifa, I., Walayat, N., Muhammad Aadil, R., Kumar, M., Wang, M., Chen, F., Cheng, K. W., & Lorenzo, J. M. (2022). Protein oxidation in muscle-

based products: Effects on physicochemical properties, quality concerns, and challenges to food industry. *Food Research International*, *157*, 111322.

Nazari, B., Mohammadifar, M. A., Shojaee-Aliabadi, S., Feizollahi, E., & Mirmoghtadaie, L. (2018). Effect of ultrasound treatments on functional properties and structure of millet protein concentrate. *Ultrasonics Sonochemistry*, *41*, 382–388.

Neji, C., Semwal, J., Kamani, M. H., Máthé, E., & Sipos, P. (2022). Legume Protein Extracts: The Relevance of Physical Processing in the Context of Structural, Techno-Functional and Nutritional Aspects of Food Development. *Processes*, *10*(12), 2586.

Nicolai, T., & Chassenieux, C. (2019). Heat-induced gelation of plant globulins. *Current Opinion in Food Science*, *27*, 18–22.

Nielsen, S. (2010). *Food Analysis* (S. Nielsen (ed.). Springer, New York.

Nieto-Nieto, T. V., Wang, Y. X., Ozimek, L., & Chen, L. (2014). Effects of partial hydrolysis on structure and gelling properties of oat globular proteins. *Food Research International*, *55*, 418–425.

Nilsson, K., Sandström, C., Özeren, H. D., Vilaplana, F., Hedenqvist, M., & Langton, M. (2022). Physiochemical and thermal characterisation of faba bean starch. *Journal of Food Measurement and Characterization*, *16*(6), 4470–4485.

Ningtyas, D. W., Tam, B., Bhandari, B., & Prakash, S. (2021). Effect of different types and concentrations of fat on the physico-chemical properties of soy protein isolate gel. *Food Hydrocolloids*, *111*, 106226.

Nishinari, K., Phillips, G. O., Nishinari, K., Fang, Y., Guo, S., & Phillips, G. O. (2014). Soy proteins: A review on composition, aggregation and emulsification.

Nivala, O., Nordlund, E., Kruus, K., & Ercili-Cura, D. (2021b). The effect of heat and transglutaminase treatment on emulsifying and gelling properties of faba bean protein isolate. *LWT*, *139*, 110517. https://doi.org/10.1016/J.LWT.2020.110517

Nosworthy, M. G., Medina, G., Franczyk, A. J., Neufeld, J., Appah, P., Utioh, A., Frohlich, P., & House, J. D. (2018). Effect of processing on the in vitro and in vivo protein quality of beans (Phaseolus vulgaris and Vicia faba). *Nutrients*, *10*(671), 1–13. Nosworthy, M. G., Medina,

G., Lu, Z. H., & House, J. D. (2023). Plant Proteins: Methods of Quality Assessment and the Human Health Benefits of Pulses. *Foods*, *12*(15), 2816. https://doi.org/10.3390/FOODS12152816

Novozymes. Viscozyme® L. Product Sheet. (2001). Retrieved February 17, 2025, from https://scholar.google.com/scholar?q=Novozymes.

O'Connor, J., Meaney, S., Williams, G. A., & Hayes, M. (2020). Extraction of protein from four different seaweeds using three different physical pre-treatment strategies. *Molecules, 25*(8), 2005.

Ochoa-Rivas, A., Nava-Valdez, Y., Serna-Saldívar, S. O., & Chuck-Hernández, C. (2017). Microwave and ultrasound to enhance protein extraction from peanut flour under alkaline conditions: Effects in yield and functional properties of protein isolates. *Food and Bioprocess Technology, 10*(3), 543-555.

O'Kane, F. E., Vereijken, J. M., Gruppen, H., & Van Boekel, M. A. J. S. (2005). Gelation Behavior of Protein Isolates Extracted from 5 Cultivars of Pisum sativum L. *Journal of Food Science*, *70*(2), C132–C137.

Olatunde, O. O., & Benjakul, S. (2018). Nonthermal Processes for Shelf-Life Extension of Seafoods: A Revisit. *Comprehensive Reviews in Food Science and Food Safety, 17*(4), 892-904.

Oluwajuyitan, T. D., & Aluko, R. E. (2024). Structural and functional properties of fava bean legumin and vicilin protein fractions. *International Journal of Food Science & Technology*, *59*(9), 6698–6716.

Opazo-Navarrete, M., Altenburg, M. D., Boom, R. M., & Janssen, A. E. M. (2018). The Effect of Gel Microstructure on Simulated Gastric Digestion of Protein Gels. *Food Biophysics,13,*124-138.

Osemwota, E. C., Alashi, A. M., Aluko, R. E., Alashi, A. M. ;, Aluko, R. E., Sadrzadeh, M., Dixon, A. G., Bazinet, L., Chae, S., & Esfahani, M. R. (2021). Comparative Study of the Structural and Functional Properties of Membrane-Isolated and Isoelectric pH Precipitated Green Lentil Seed Protein Isolates. *Membranes, 11*(9), 694.

Paiva, A., Craveiro, R., Aroso, I., Martins, M., Reis, R. L., & Duarte, A. R. C. (2014). Natural deep eutectic solvents - Solvents for the 21st century. *ACS Sustainable Chemistry and Engineering*, *2*(5), 1063–1071.

Pan, M., Xu, F., Wu, Y., Yao, M., Xiao, X., Zhang, N., Ju, X., & Wang, L. (2020). Application of ultrasound-assisted physical mixing treatment improves in vitro protein digestibility of rapeseed napin. *Ultrasonics Sonochemistry, 67*, 105136.

Pangestuti, R., Getachew, A. T., Siahaan, E. A., & Chun, B.-S. (2019). Characterization of functional materials derived from tropical red seaweed *Hypnea musciformis* produced by subcritical water extraction systems. *Journal of Applied Phycology, 31*(4), 2517-2528.

Paredes-López, O., Ordorica-Falomir, C., & Olivares-Vázquez, M. R. (1991). Chickpea Protein Isolates: Physicochemical, Functional and Nutritional Characterization. *Journal of Food Science*, *56*(3), 726–729.

Park, J.-S., Jeong, Y.-R., & Chun, B.-S. (2019). Physiological activities and bioactive compound from laver (*Pyropia yezoensis*) hydrolysates by using subcritical water hydrolysis. *The Journal of Supercritical Fluids, 148*, 130-136.

Pawar, S. S., Iyyaswami, R., & Belur, P. D. (2017). Reverse micellar extraction of lactoferrin from its synthetic solution using CTAB/n-heptanol system. *Journal of Food Science and Technology, 54*(11), 3630-3639.

Pearce, K. N., & Kinsella, J. E. (1978). Emulsifying Properties of Proteins: Evaluation of a Turbidimetric Technique. *Journal of Agricultural and Food Chemistry*, *26*(3), 716–723.

Pelgrom, P. J. M., Vissers, A. M., Boom, R. M., & Schutyser, M. A. I. (2013). Dry fractionation for production of functional pea protein concentrates. *Food Research International, 53*(1), 232-239.

Penha, C., Falcão, H., Ida, E., Speranzo, P., & Kurozawa, L.E. (2020). Enzymatic pretreatment in the extraction process of soybean to improve protein and isoflavone recovery and to favor aglycone formation. *Food research International, 137*, 190624.

Pereira, K. A. B., Oliveira, P. F., Chaves, I., Pedroni, L. G., Oliveira, L. A., & Mansur, C. R. E. (2022). Rheological properties of nanocomposite hydrogels containing aluminum and zinc oxides with potential application for conformance control. *Colloid and Polymer Science*, *300*(6), 609–624.

Perkins, S. L., Painter, P., & Colina, C. M. (2014). Experimental and computational studies of choline chloride-based deep eutectic solvents. *Journal of Chemical and Engineering Data*, *59*(11), 3652–3662.

Perović, M. N., Knežević Jugović, Z. D., & Antov, M. G. (2020). Improved recovery of protein from soy grit by enzyme-assisted alkaline extraction. *Journal of Food Engineering, 276*, 109894.

Petitot, M., Boyer, L., Minier, C., & Micard, V. (2010). Fortification of pasta with split pea and faba bean flours: Pasta processing and quality evaluation. *Food Research International*, *43*(2), 634–641.

Phongthai, S., Lim, S.-T., & Rawdkuen, S. (2016). Optimization of microwave-assisted extraction of rice bran protein and its hydrolysates properties. *Journal of Cereal Science, 70*, 146-154.

Picó, Y. (2013). Ultrasound-assisted extraction for food and environmental samples. *TrAC Trends in Analytical Chemistry, 43*, 84-99.

Pierucci, S., Klemeš, J. J., Piazza, L., Bakalis, S., Cicci, A., Sed, G., & Bravi, M. (2018). Potential of choline chloride - based natural deep eutectic solvents (NaDES) in the extraction of microalgal metabolites. *Chemical Engineering Transactions*, *57*(2017), 61–66.

PIMA. (2017). Protein Ingredient Market Analysis: New market reports.

Pimentel-Moral, S., Borrás-Linares, I., Lozano-Sánchez, J., Arráez-Román, D., Martínez-Férez, A., & Segura-Carretero, A. (2018). Microwave-assisted extraction for *Hibiscus sabdariffa* bioactive compounds. *Journal of Pharmaceutical and Biomedical Analysis, 156*, 313-322.

Pingret, D., Fabiano-Tixier, A. S., & Chemat, F. (2013). Degradation during application of ultrasound in food processing: A review. *Food Control*, *31*(2), 593–606.

Plaza, M., & Turner, C. (2015). Pressurized hot water extraction of bioactives. *TrAC Trends in Analytical Chemistry, 71*, 39-54.

Plazzotta, S., Moretton, M., Calligaris, S., & Manzocco, L. (2021). Physical, chemical, and techno-functional properties of soy okara powders obtained by high pressure homogenization and alkaline-acid recovery. *Food and Bioproducts Processing, 128*, 95-101.

Pojić, M., Mišan, A., & Tiwari, B. (2018). Eco-innovative technologies for extraction of proteins for human consumption from renewable protein sources of plant origin. In *Trends in Food Science and Technology, 75,* 93–104.

Polikovsky, M., Fernand, F., Sack, M., Frey, W., Müller, G., & Golberg, A. (2016). Towards marine biorefineries: Selective proteins extractions from marine macroalgae Ulva with pulsed electric fields. *Innovative Food Science and Emerging Technologies, 37*, 194-200.

Polikovsky, M., Fernand, F., Sack, M., Frey, W., Müller, G., & Golberg, A. (2019). In silico food allergenic risk evaluation of proteins extracted from macroalgae *Ulva sp.* with pulsed electric fields. *Food Chemistry, 276*, 735-744.

Pozo, C., Rodríguez-Llamazares, S., Bouza, R., Barral, L., Castaño, J., Müller, N., & Restrepo, I. (2018.). Study of the structural order of native starch granules using combined FTIR and XRD analysis, *Journal of Polymer Reaserch, 25*, 266.

Prabhu, M. S., Levkov, K., Livney, Y. D., Israel, A., & Golberg, A. (2019). High-voltage pulsed electric field preprocessing enhances extraction of starch, proteins, and ash from marine macroalgae *Ulva ohnoi*. *ACS Sustainable Chemistry & Engineering, 7*(20), 17453-17463.

Prabhune, A., & Dey, R. (2023). Green and sustainable solvents of the future: Deep eutectic solvents. *Journal of Molecular Liquids*, *379*, 121676.

Pradeepkumar, P., Subbiah, A., & Rajan, M. (2019). Synthesis of bio-degradable poly(2-hydroxyethyl methacrylate) using natural deep eutectic solvents for sustainable cancer drug delivery. *SN Applied Sciences*, *1*(6), 1–13.

Pulkkinen, M., Coda, R., Lampi, A. M., Varis, J., Katina, K., & Piironen, V. (2019). Possibilities of reducing amounts of vicine and convicine in faba bean suspensions and sourdoughs. *European Food Research and Technology*, *245*(7), 1507–1518.

Puranik, S., Sanjay Pai, P., & Rao, G. (2009). Determination of organic volatile impurities in herbal formulations and extracts by capillary gas chromatography. *International Journal of Applied Research in Natural Products, 2*(1), 32-46.

Qian, C., Decker, E. A., Xiao, H., & McClements, D. J. (2012). Physical and chemical stability of

β-carotene-enriched nanoemulsions: Influence of pH, ionic strength, temperature, and emulsifier type. *Food Chemistry*, *132*(3), 1221–1229.

Qiao, Q., Shi, J., & Shao, Q. (2021). Effects of water on the solvation and structure of lipase in deep eutectic solvents containing a protein destabilizer and stabilizer. *Physical Chemistry Chemical Physics*, *23*(40), 23372–23379.

Rahman, M. M., Byanju, B., Grewell, D., & Lamsal, B. P. (2020). High-power sonication of soy proteins: Hydroxyl radicals and their effects on protein structure. *Ultrasonics Sonochemistry*, *64*, 105019.

Rahman, M. M., & Lamsal, B. P. (2021). Ultrasound-assisted extraction and modification of plant-based proteins: Impact on physicochemical, functional, and nutritional properties. *Comprehensive Reviews in Food Science and Food Safety*, *20*(2), 1457–1480.

Rahman, M. M., & Lamsal, B. P. (2023). Effects of atmospheric cold plasma and high-power sonication on rheological and gelling properties of mung bean protein dispersions. *Food Research International*, *163*, 112265.

Raikos, V., Neacsu, M., Russell, W., & Duthie, G. (2014). Comparative study of the functional properties of lupin, green pea, fava bean, hemp, and buckwheat flours as affected by pH. *Food Science and Nutrition*, *2*(6), 802–810.

Ranjha, M. M. A. N., Kanwal, R., Shafique, B., Arshad, R. N., Irfan, S., Kieliszek, M., Kowalczewski, P. Ł., Irfan, M., Khalid, M. Z., & Roobab, U. (2021). A critical review on pulsed electric field: A novel technology for the extraction of phytoconstituents. *Molecules, 26*(16), 4893.

Ravber, M., Knez, Ž., & Škerget, M. (2015). Simultaneous extraction of oil- and water-soluble phase from sunflower seeds with subcritical water. *Food Chemistry, 166*, 316-323.

Reis, M. F. T., Bonomo, R. C. F., de Sousa, R. d. C. S., Veloso, C. M., Fontan, R. d. C. I., & Gandolfi, O. R. R. (2016). Optimization of protein extraction process from jackfruit seed flour by reverse micelle system. *Acta Scientiarum. Technology, 38*(3), 283-290.

Rempel, C., Geng, X., & Zhang, Y. (2019). Industrial scale preparation of pea flour fractions with enhanced nutritive composition by dry fractionation. *Food Chemistry, 276*, 119-128.

Resendiz-Vazquez, J. A., Ulloa, J. A., Urías-Silvas, J. E., Bautista-Rosales, P. U., Ramírez-Ramírez, J. C., Rosas-Ulloa, P., & González-Torres, L. (2017). Effect of high-intensity ultrasound on the technofunctional properties and structure of jackfruit (Artocarpus heterophyllus) seed protein isolate. *Ultrasonics Sonochemistry*, *37*, 436–444.

Ricci, L., Umiltà, E., Righetti, M. C., Messina, T., Zurlini, C., Montanari, A., Bronco, S., & Bertoldo, M. (2018). On the thermal behavior of protein isolated from different legumes investigated by DSC and TGA. *Journal of the Science of Food and Agriculture*, *98*(14), 5368–5377.

Rose, J. K. C. (2004). The Plant Cell Wall. *Annals of botany, 94*(4), 645.

Roslan, M. A. M., Sobri, Z. M., Zuan, A. T. K., Cheak, S. C., & Rahman, N. A. A. (2022). Bioprospecting microwave-alkaline hydrolysate cocktail of defatted soybean meal and jackfruit peel biomass as carrier additive of molasses-alginate-bead biofertilizer. *Scientific reports, 12*(1), 1-14.

Rosset, M., Acquaro, V. R., & Beléia, A. D. P. (2014). Protein extraction from defatted soybean flour with Viscozyme L pretreatment. *Journal of Food Processing and Preservation*, *38*(3), 784–790.

Sá, A. G. A., Hang, J., Jardine, L., Bett, K. E., & House, J. D. (2023). How different amino acid scoring patterns recommended by FAO/WHO can affect the nutritional quality and protein claims of lentils. *Sustainable Food Proteins*, *1*(2), 59–73.

Sá, A. G. A., Moreno, Y. M. F., & Carciofi, B. A. M. (2019). Food processing for the improvement of plant proteins digestibility. *Critical Reviews in Food Science and Nutrition*, *60*(20), 3367–3386.

Sá, A. G. A., Moreno, Y. M. F., & Carciofi, B. A. M. (2020). Plant proteins as high-quality nutritional source for human diet. *Trends in Food Science & Technology*, *97*, 170–184.

Sá, A. G. A., Pacheco, M. T. B., Moreno, Y. M. F., & Carciofi, B. A. M. (2022). Cold-pressed sesame seed meal as a protein source: Effect of processing on the protein digestibility, amino acid profile, and functional properties. *Journal of Food Composition and Analysis, 111*, 104634.

Sahni, P., Sharma, S., & Surasani, V. K. R. (2020). Influence of processing and pH on amino acid profile, morphology, electrophoretic pattern, bioactive potential and functional characteristics of alfalfa protein isolates. *Food Chemistry*, *333*, 127503.

Salvador-Reyes, R., Furlan, L. C., Martínez-Villaluenga, C., Martins Dala-Paula, B., Pedrosa, M. T., & Clerici, S. (2023). From ancient crop to modern superfood: Exploring the history, diversity, characteristics, technological applications, and culinary uses of Peruvian fava

beans. *Food Research International*, *173*, 963–9969.

San, Y., Xing, Y., Li, B., & Zheng, L. (2025). Effect of transglutaminase cross-linking on the structure and emulsification performance of heated black bean protein isolate. *Journal of the Science of Food and Agriculture*, *105*(4), 2382–2389.

Sanchez-Fernandez, A., Hammond, O. S., Jackson, A. J., Arnold, T., Doutch, J., & Edler, K. J. (2017). Surfactant-Solvent Interaction Effects on the Micellization of Cationic Surfactants in a Carboxylic Acid-Based Deep Eutectic Solvent. *Langmuir*, *33*(50), 14304–14314.

Sanchez-Fernandez, A., & Jackson, A. J. (2021). Proteins in deep eutectic solvents: Structure, dynamics and interactions with the solvent. *Advances in Botanical Research, 97,* 69-94.

Sánchez-Vega, R., Elez-Martínez, P., & Martín-Belloso, O. (2015). Influence of high-intensity pulsed electric field processing parameters on antioxidant compounds of broccoli juice. *Innovative Food Science and Emerging Technologies, 29*, 70-77.

Sankaran, R., Bong, J. H., Chow, Y. H., Wong, F. W. F., Ling, T. C., & Show, P. L. (2019). Reverse micellar system in protein recovery - A review of the latest developments. *Current Protein & Peptide Science 20*(10), 1012-1026.

Sapir, L., & Harries, D. (2020). Restructuring a Deep Eutectic Solvent by Water: The Nanostructure of Hydrated Choline Chloride/Urea. *Journal of Chemical Theory and Computation*, *16*(5), 3335–3342.

Saravana, P. S., Choi, J. H., Park, Y. B., Woo, H. C., & Chun, B. S. (2016). Evaluation of the chemical composition of brown seaweed (*Saccharina japonica*) hydrolysate by pressurized

hot water extraction. *Algal Research, 13*, 246-254.

Sari, Y. W., Bruins, M. E., & Sanders, J. P. M. (2013). Enzyme assisted protein extraction from rapeseed, soybean, and microalgae meals. *Industrial Crops and Products, 43*, 78-83.

Sarkar, S., Ghosh, S., & Chakrabarti, R. (2017). Ammonium based stabilizers effectively counteract urea-induced denaturation in a small protein: insights from molecular dynamics simulations. *RSC Advances, 7*, 52888-52906.

Sathe, S. K., Deshpande, S. S., & Salunkhe, D. K. (1982). Functional Properties of Winged Bean [Psophocarpus tetragonolobus (L.) DC] Proteins. *Journal of Food Science*, *47*(2), 503–509.

Schutyser, M. A. I., Pelgrom, P. J. M., van der Goot, A. J., & Boom, R. M. (2015). Dry fractionation for sustainable production of functional legume protein concentrates. *Trends in Food Science and Technology, 45*(2), 327-335.

Shah, D., & Mjalli, F. S. (2014). Effect of water on the thermo-physical properties of Reline: An experimental and molecular simulation based approach. *Physical Chemistry Chemical Physics*, *16*(43), 23900–23907.

Shan, H., Lu, S. W., Jiang, L. Z., Wang, L. K., Liao, H., Zhang, R. Y., Dai, C. J., Yao, X. M., Zhang, Y. L., Su, P., & Sun, X. D. (2015). Gelation Property of Alcohol-Extracted Soy Protein Isolate and Effects of Various Reagents on the Firmness of Heat-Induced Gels. *International Journal of Food Properties*, *18*(3), 627–637.

Sharan, S., Zanghelini, G., Zotzel, J., Bonerz, D., Aschoff, J., Saint-Eve, A., & Maillard, M.-N. (2021). Fava bean (Vicia faba L.) for food applications: From seed to ingredient processing

and its effect on functional properties, antinutritional factors, flavor, and color. *Comprehensive Reviews in Food Science and Food Safety*, *20*(1), 401–428.

Sharan, S., Zotzel, J., Stadtmüller, J., Bonerz, D., Aschoff, J., Olsen, K., Rinnan, Å., Saint-Eve, A., Maillard, M. N., & Orlien, V. (2022). Effect of industrial process conditions of fava bean (Vicia faba L.) concentrates on physico-chemical and functional properties. *Innovative Food Science & Emerging Technologies*, *81*, 103142.

Sharma, V., Tsai, M. L., Sun, P. P., Chen, C. W., Nargotra, P., & Dong, C. Di. (2023). Sequential ultrasound assisted deep eutectic solvent-based protein extraction from Sacha inchi meal biomass: towards circular bioeconomy. *Journal of Food Science and Technology*, *60*(4), 1425–1434.

Shen, P., Peng, J., Sagis, L. M. C., & Landman, J. (2024). Molecular, interfacial and foaming properties of pulse proteins. *Food Hydrocolloids*, *156*, 110313.

Shen, S., Zhou, C., Zeng, Y., Zhang, H., Hossen, M. A., Dai, J., Li, S., Qin, W., & Liu, Y. (2022). Structures, physicochemical and bioactive properties of polysaccharides extracted from Panax notoginseng using ultrasonic/microwave-assisted extraction. *LWT*, *154*, 112446.

Shen, Y., Du, Z., Wu, X., & Li, Y. (2022). Modulating molecular interactions in pea protein to improve its functional properties. *Journal of Agriculture and Food Research*, *8*, 100313.

Shevkani, K., Singh, N., Chen, Y., Kaur, A., & Yu, L. (2019). Pulse proteins: secondary structure, functionality and applications. *Journal of Food Science and Technology*, *56*(6), 2787.

Shevkani, K., Singh, N., Kaur, A., & Rana, J. C. (2015). Structural and functional characterization of kidney bean and field pea protein isolates: A comparative study. *Food Hydrocolloids*, *43*, 679–689.

Shi, D., & Nickerson, M. T. (2022). Comparative evaluation of the functionality of faba bean protein isolates with major legume proteins in the market. *Cereal Chemistry*, *99*(6), 1246–1260.

Sierra, L. S., Dixon, C. K., & Wilken, L. R. (2017). Enzymatic cell disruption of the microalgae *Chlamydomonas reinhardtii* for lipid and protein extraction. *Algal Research, 25*, 149-159.

Silva-Sanchez, C., Li, H., & Chen, S. (2015). Recent advances and challenges in plant phosphoproteomics. *Proteomics, 15*(5-6), 1127-1141.

Silva, S. C., Almeida, T., Colucci, G., Santamaria-Echart, A., Manrique, Y. A., Dias, M. M., Barros, L., Fernandes, Â., Colla, E., & Barreiro, M. F. (2022). Spirulina (*Arthrospira platensis*) protein-rich extract as a natural emulsifier for oil-in-water emulsions: Optimization through a sequential experimental design strategy. *Colloids and Surfaces A: Physicochemical and Engineering Aspects, 648*, 129264.

Sim, S. Y. J., Srv, A., Chiang, J. H., & Henry, C. J. (2021). Plant Proteins for Future Foods: A Roadmap. *Foods*, *10*(8), 1967.

Singhal, A., Karaca, A. C., Tyler, R., & Nickerson, M. (2016). Garin legumes. In A.K. Goyal (Eds.), *Pulse proteins: from processing to structure-function relationships* (pp.55). IntechOpen.

Skulcova, A., Russ, A., Jablonsky, M., & Sima, J. (2018). The pH Behavior of Seventeen Deep Eutectic Solvents. *BioResources*, *13*(3), 5042–5051.

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

Sofía, A., María, G., Ana, A., & Epameinondas, X. (2021). Potential of Wastewater Valorization after Wet Extraction of Proteins from Faba Bean and Pea Flours. *Recent Progress in Materials*, *3*(2), 1–1.

Stefanovic, R., Ludwig, M., Webber, G. B., Atkin, R., & Page, A. J. (2017). Nanostructure, hydrogen bonding and rheology in choline chloride deep eutectic solvents as a function of the hydrogen bond donor. *Physical Chemistry Chemical Physics*, *19*(4), 3297–3306.

Stone, A. K., Karalash, A., Tyler, R. T., Warkentin, T. D., & Nickerson, M. T. (2014). Functional Attributes of Pea Protein Isolates Prepared Using Different Extraction Methods and Cultivars. Food Research International*, 76* (1), 31-38.

Subaşı, B. G., Casanova, F., Güven, E. Ç., & Mohammadifar, M. A. (2019). Effect of removing phenolic compounds on interfacial behavior of protein isolated from de-oiled sunflower cake. In *8th International Symposium on Food Rheology and Structure - ISFRS*). Zürich, Switzerland.

Suchintita Das, R., Zhu, X., Hannon, S., Mullins, E., Alves, S., Garcia-Vaquero, M., & Tiwari, B. K. (2023). Exploring Osborne fractionation and laboratory/pilot scale technologies (conventional extraction, ultrasound-assisted extraction, high-pressure processing and hydrodynamic cavitation) for protein extraction from faba bean (Vicia faba L.). *Innovative*

Food Science & Emerging Technologies, *89*, 103487.

Sui, X., Zhang, T., & Jiang, L. (2021). Soy Protein: Molecular Structure Revisited and Recent Advances in Processing Technologies. *Annual Review of Food Science and Technology*, *12*, 119–147.

Sun, H., Xin, R., & Qu, D., & Yao, F. (2020). Mechanism of deep eutectic solvents enhancing catalytic function of cytochrome P450 enzymes in biosynthesis and organic synthesis. *Journal of Biotechnology,323,* 264-273.

Sun, X., & Bandara, N. (2019). Applications of reverse micelles technique in food science: A comprehensive review. In *Trends in Food Science and Technology, 91*, 106–115).

Sun, X. D., & Arntfield, S. D. (2010). Gelation properties of salt-extracted pea protein induced by heat treatment. *Food Research International*, *43*(2), 509–515.

Sun, X. D., & Arntfield, S. D. (2012). Molecular forces involved in heat-induced pea protein gelation: Effects of various reagents on the rheological properties of salt-extracted pea protein gels. *Food Hydrocolloids*, *28*(2), 325–332.

Sun, X.-H., Zhu, K.-X., & Zhou, H.-M. (2008). Protein extraction from defatted wheat germ by reverse micelles: Optimization of the forward extraction. *Journal of Cereal Science, 48*(3), 829-835.

Sunphorka, S., Chavasiri, W., Oshima, Y., & Ngamprasertsith, S. (2012). Protein and sugar extraction from rice bran and de-oiled rice bran using subcritical water in a semi-

continuous reactor: optimization by response surface methodology. *International Journal of Food Engineering, 8*(3).

Švarc-Gajić, J., Morais, S., Delerue-Matos, C., Vieira, E. F., & Spigno, G. (2020). Valorization potential of oilseed cakes by subcritical water extraction. *Applied Sciences, 10*(24), 8815.

Tang, C. H., & Sun, X. (2011). A comparative study of physicochemical and conformational properties in three vicilins from Phaseolus legumes: Implications for the structure–function relationship. *Food Hydrocolloids*, *25*(3), 315–324.

Tang, C. H., Wang, X. Y., Yang, X. Q., & Li, L. (2009). Formation of soluble aggregates from insoluble commercial soy protein isolate by means of ultrasonic treatment and their gelling properties. *Journal of Food Engineering*, *92*(4), 432–437.

Tang, D. S., Tian, Y.-J., He, Y. Z., Li, L., Hu, S. Q., & Li, B. (2010). Optimisation of Ultrasonic-assisted Protein Extraction from Brewer's Spent Grain. *Czech Journal of Food Science*, *28*(1), 9–17.

Tang, Q., Roos, Y. H., Vahedikia, N., & Miao, S. (2024). Evaluation on pH-dependent thermal gelation performance of chickpea, pea protein, and casein micelles. *Food Hydrocolloids*, *149*, 109618.

Tang, X., Shen, Y., Zhang, Y., Schilling, M., Li, Y. (2021). Parallel comparison of functional and physicochemical properties of common pulse proteins. *LWT, 146,* 11594.

Tanger, C., Engel, J., & Kulozik, U. (2020). Influence of extraction conditions on the conformational alteration of pea protein extracted from pea flour. *Food Hydrocolloids*, *107*,

105949.

Tanger, C., Müller, M., Andlinger, D., & Kulozik, U. (2022). Influence of pH and ionic strength on the thermal gelation behaviour of pea protein. *Food Hydrocolloids*, *123*, 106903.

Teo, C. C., Tan, S. N., Yong, J. W. H., Hew, C. S., & Ong, E. S. (2010). Pressurized hot water extraction (PHWE). *Journal of Chromatography A, 1217*(16), 2484-2494.

Thakur, A., Verma, M., Bharti, R., & Sharma, R. (2022). Recent Advances in Utilization of Deep Eutectic Solvents: An Environmentally Friendly Pathway for Multi-component Synthesis. *Current Organic Chemistry*, *26*(3), 299–323.

Tian, Y., Kriisa, M., Föste, M., Kütt, M. L., Zhou, Y., Laaksonen, O., & Yang, B. (2022). Impact of enzymatic pre-treatment on composition of nutrients and phytochemicals of canola (Brassica napus) oil press residues. *Food Chemistry*, *387*, 132911.

Tirgar, M., Silcock, P., Carne, A., & Birch, E. J. (2017). Effect of extraction method on functional properties of flaxseed protein concentrates. *Food Chemistry, 215*, 417-424.

Tinus, T., Damour, M., Van Riel, V., & Sopade, P. A. (2012). Particle size-starch-protein digestibility relationships in cowpea (Vigna unguiculata). *Journal of Food Engineering*, *113*(2), 254–264.

Tomé, L. I. N., Baião, V., da Silva, W., & Brett, C. M. A. (2018). Deep eutectic solvents for the production and application of new materials. *Applied Materials Today*, *10*, 30–50.

Tsermoula, P., Virgili, C., Ortega, R. G., Mullen, A. M., Álvarez, C., O'Brien, N. M., O'Flaherty, E. A. A., & O'Neill, E. E. (2019). Functional protein rich extracts from bovine and porcine

hearts using acid or alkali solubilisation and isoelectric precipitation. *International Journal of Food Science and Technology, 54*(4), 1292-1298.

Turker, I., & Isleroglu, H. (2024). Ultrasound and enzymatic treatments to improve protein extraction from cress seeds, and the characterization of protein isolates. *Food Bioscience*, *60*, 104443.

UN. (2017). United Nations: World Population Prospects: The 2017 Revision. New York.

Uruakpa, F. O. (2012). Gelling Behavior of Plant Proteins and Polysaccharides in Food Systems. *Journal of Food Science and Engineering*, *2*, 247–256.

Valente, I. M., Cabrita, A. R. J., Malushi, N., Oliveira, H. M., Papa, L., Rodrigues, J. A., Fonseca, A. J. M., & Maia, M. R. G. (2018). Unravelling The Phytonutrients and Antioxidant Properties of European Vicia Faba L. Seeds. *Food research international, 116*, 888-896.

Valenzuela, C., Abugoch, L., Tapia, C., & Gamboa, A. (2013). Effect of alkaline extraction on the structure of the protein of quinoa (*Chenopodium quinoa* Willd.) and its influence on film formation. *International Journal of Food Science & Technology, 48*(4), 843-849.

Van Kleef, F. S. M. (1986). Thermally induced protein gelation: Gelation and rheological characterization of highly concentrated ovalbumin and soybean protein gels. *Biopolymers*, *25*(1), 31–59.

Van Loo, E. J., Hoefkens, C., & Verbeke, W. (2017). Healthy, sustainable and plant-based eating: Perceived (mis)match and involvement-based consumer segments as targets for future policy. *Food Policy, 69*, 46-57.

Van Osch, D. J. G. P., Dietz, C. H. J. T., Van Spronsen, J., Kroon, M. C., Gallucci, F., Van Sint Annaland, M., & Tuinier, R. (2019). A Search for Natural Hydrophobic Deep Eutectic Solvents Based on Natural Components. *ACS Sustainable Chemistry and Engineering*, *7*(3), 2933–2942.

Varghese, T., & Pare, A. (2019). Effect of microwave assisted extraction on yield and protein characteristics of soymilk. *Journal of Food Engineering, 262*, 92-99.

Verfaillie, D., Janssen, F., Van Royen, G., & Wouters, A. G. B. (2023). A systematic study of the impact of the isoelectric precipitation process on the physical properties and protein composition of soy protein isolates. *Food Research International*, *163*, 112177.

Vilková, M., Płotka-Wasylka, J., & Andruch, V. (2020). The role of water in deep eutectic solvent-base extraction. *Journal of Molecular Liquids*, *304*, 112747.

Vioque, J., Alaiz, M., & Girón-Calle, J. (2012). Nutritional and functional properties of Vicia faba protein isolates and related fractions. *Food Chemistry*, *132*(1), 67–72.

Visschers, R. W., & De Jongh, H. H. J. (2005). Disulphide bond formation in food protein aggregation and gelation. *Biotechnology Advances*, *23*(1), 75–80.

Vogelsang-O'Dwyer, M., Petersen, I. L., Joehnke, M. S., Sørensen, J. C., Bez, J., Detzel, A., Busch, M., Krueger, M., O'Mahony, J. A., Arendt, E. K., & Zannini, E. (2020). Comparison of Faba Bean Protein Ingredients Produced Using Dry Fractionation and Isoelectric Precipitation: Techno-Functional, Nutritional and Environmental Performance. *Foods*, *9*(3).

Wahlström, R., Rommi, K., Willberg-Keyriläinen, P., Ercili-Cura, D., Holopainen-Mantila, U.,

Hiltunen, J., Mäkinen, O., Nygren, H., Mikkelson, A., & Kuutti, L. (2017). High yield protein extraction from brewer's spent grain with novel carboxylate salt - Urea aqueous deep eutectic solvents. *Chemistry Select, 2*(29), 9355-9363.

Wang, F., Zhang, Y., Xu, L., & Ma, H. (2020). An efficient ultrasound-assisted extraction method of pea protein and its effect on protein functional properties and biological activities. *LWT*, *127*, 109348.

Wang, H., Tong, X., Yuan, Y., Peng, X., Zhang, Q., Zhang, S., Xie, C., Zhang, X., Yan, S., Xu, J., Jiang, L., Qi, B., & Li, Y. (2020). Effect of Spray-Drying and Freeze-Drying on the Properties of Soybean Hydrolysates. *Journal of Chemistry*, *2020*.

Wang, Q., Wang, Y., Huang, M., Hayat, K., Kurtz, N. C., Wu, X., Ahmad, M., & Zheng, F. (2021). Ultrasound-assisted alkaline proteinase extraction enhances the yield of pecan protein and modifies its functional properties. *Ultrasonics Sonochemistry*, *80*, 105789.

Wang, X., He, Z., Zeng, M., Qin, F., Adhikari, B., & Chen, J. (2017). Effects of the size and content of protein aggregates on the rheological and structural properties of soy protein isolate emulsion gels induced by CaSO4. *Food Chemistry*, *221*, 130–138.

Wang, Y., Wang, Z., Handa, C. L., & Xu, J. (2017). Effects of ultrasound pre-treatment on the structure of β-conglycinin and glycinin and the antioxidant activity of their hydrolysates. *Food Chemistry*, *218*, 165–172.

Wang, Z., Li, Y., Jiang, L., Qi, B., & Zhou, L. (2014). Relationship between secondary structure and surface hydrophobicity of soybean protein isolate subjected to heat treatment. *Journal of Chemistry*, *2014* (1).

Wang, Z., Zhao, X., Hu, H., Wang, M., Zhang, X., & Liu, H. (2021). Improved backward extraction of walnut protein using AOT reverse micelles with microwave and its characteristics. *Journal of Food Processing and Preservation, 45*(6), e15470.

Wanjun, X., Qingqing, Y., Yaqi, Z., Yu, F., Qiaoyu, C., Ruichang, G., & Yulong, B. (2024). Insight into the mechanism of the decrease in mechanical strength and water-holding capacity of gels made from oxidized gelatin. *International Journal of Biological Macromolecules*, *258*, 128842.

Warren, F. J., Gidley, M. J., & Flanagan, B. M. (2016). Infrared spectroscopy as a tool to characterise starch ordered structure—a joint FTIR–ATR, NMR, XRD and DSC study. *Carbohydrate Polymers*, *139*, 35–42.

Warsame, A., Michael, N., O' Sullivan, D. M., & Tosi, P. (2020). Identification and quantification of major faba bean seed proteins. *ACS Publications*, *68*(32), 8544.

Wazeer, I., Hayyan, M., & Hadj-Kali, M. K. (2018). Deep eutectic solvents: designer fluids for chemical processes. *Journal of Chemical Technology & Biotechnology*, *93*(4), 945–958.

Wei, C.-L., Lu, W., Yang, J., Wang, M.-P., Yang, X.-Q., & Wang, J.-M. (2018). Physicochemical properties of soy protein prepared by enzyme-assisted countercurrent extraction. *International Journal of Food Science and Technology, 53*(6), 1389-1396.

Wen, C., Zhang, J., Yao, H., Zhou, J., Duan, Y., Zhang, H., & Ma, H. (2019). Advances in renewable plant-derived protein source: The structure, physicochemical properties affected by ultrasonication. *Ultrasonics Sonochemistry*, *53*, 83–98.

Wen, L., Álvarez, C., Zhang, Z., Poojary, M. M., Lund, M. N., Sun, D.-W., & Tiwari, B. K. (2021). Optimisation and characterisation of protein extraction from coffee silverskin assisted by ultrasound or microwave techniques. *Biomass Conversion and Biorefinery, 11*(5), 1575-1585.

Wiboonsirikul, J., & Adachi, S. (2008). Extraction of functional substances from agricultural products or by-products by subcritical water treatment. *Food Science and Technology Research, 14*(4), 319-319.

Wijngaard, H., Hossain, M. B., Rai, D. K., & Brunton, N. (2012). Techniques to extract bioactive compounds from food by-products of plant origin. *Food Research International, 46*(2), 505-513.

Williamson, S. T., Shahbaz, K., Mjalli, F. S., AlNashef, I. M., & Farid, M. M. (2017). Application of deep eutectic solvents as catalysts for the esterification of oleic acid with glycerol. *Renewable Energy*, *114*, 480–488.

Wong, F. W. F., Ariff, A. B., & Stuckey, D. C. (2018). Downstream protein separation by surfactant precipitation: a review. *Critical Reviews in Biotechnology, 38*(1), 31-46.

Wright, D. J., & Boulter, D. (1974). Purification and subunit structure of legumin of Vicia faba L. (broad bean). *Biochemical Journal*, *141*(2), 413–418.

Wu, C., Ma, W., Chen, Y., Navicha, W. B., Wu, D., & Du, M. (2019). The water holding capacity and storage modulus of chemical cross-linked soy protein gels directly related to aggregates size. *LWT*, *103*, 125–130.

Xi, J., Li, Z., & Fan, Y. (2021). Recent advances in continuous extraction of bioactive ingredients from food-processing wastes by pulsed electric fields. *Critical Reviews in Food Science and Nutrition, 61*(10), 1738-1750.

Xing, Q., de Wit, M., Kyriakopoulou, K., Boom, R. M., & Schutyser, M. A. I. (2018). Protein enrichment of defatted soybean flour by fine milling and electrostatic separation. *Innovative Food Science and Emerging Technologies, 50*, 42-49.

Xing, Q., Dekker, S., Kyriakopoulou, K., Boom, R. M., Smid, E. J., & Schutyser, M. A. I. (2020). Enhanced nutritional value of chickpea protein concentrate by dry separation and solid state fermentation. *Innovative Food Science and Emerging Technologies, 59*, 102269.

Xiong, Y. L., & Guo, A. (2020). Animal and Plant Protein Oxidation: Chemical and Functional Property Significance. *Foods*, *10*(1), 40.

Xu, G. C., Ding, J. C., Han, R. Z., Dong, J. J., & Ni, Y. (2016). Enhancing cellulose accessibility of corn stover by deep eutectic solvent pretreatment for butanol fermentation. *Bioresource Technology*, *203*, 364–369.

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, 864*, 9-20.

Xu, Y., Xia, W., Yang, F., & Nie, X. (2010). Protein molecular interactions involved in the gel network formation of fermented silver carp mince inoculated with Pediococcus pentosaceus. *Food Chemistry*, *120 (*3*)*, 717-723.

Yang, C., Liu, W., Zhu, X., Zhang, X., Wei, Y., Huang, J., Yang, F., & Yang, F. (2024).

Ultrasound-assisted enzymatic digestion for efficient extraction of proteins from quinoa. *LWT*, *194*, 115784.

Yang, H., Yang, S., Kong, J., Dong, A., and Yu, S. (2015). Obtaining information about protein secondary structures in aqueous solution using Fourier transform IR spectroscopy. *Nature Protocol, 10,* 382-396.

Yang, H., Yang, S., Kong, J., Dong, A., & Yu, S. (2015). Obtaining information about protein secondary structures in aqueous solution using Fourier transform IR spectroscopy. *Nature Protocols*, *10*(3), 382–396.

Yang, J., Kornet, R., Diedericks, C. F., Yang, Q., Berton-Carabin, C. C., Nikiforidis, C. V., Venema, P., van der Linden, E., & Sagis, L. M. C. (2022). Rethinking plant protein extraction: Albumin—From side stream to an excellent foaming ingredient. *Food Structure*, *31*, 100254.

Yang, J., Liu, G., Zeng, H., & Chen, L. (2018). Effects of high pressure homogenization on faba bean protein aggregation in relation to solubility and interfacial properties. *Food Hydrocolloids*, *83*, 275–286.

Yang, J., & Sagis, L. M. C. (2021). Interfacial behavior of plant proteins — novel sources and extraction methods. *Current Opinion in Colloid & Interface Science*, *56*, 101499.

Yang, J., Zamani, S., Liang, L., & Chen, L. (2021). Extraction methods significantly impact pea protein composition, structure and gelling properties. *Food Hydrocolloids*, *117*, 106678.

Yang, X., Su, Y., & Li, L. (2020). Study of soybean gel induced by Lactobacillus plantarum: Protein structure and intermolecular interaction. *LWT*, *119*, 108794.

Yao, F., Chen, F.-s., Du, Y., Zhang, Q., & Zhu, T.-w. (2021). Functional and structural properties of soy 11S globulin: Influence of reverse micelle extraction. *Journal of Food Science, 86*(8), 3403-3412.

Yu, D., Xue, Z., & Mu, T. (2021). Eutectics: Formation, properties, and applications. *Chemical Society Reviews*, *50*(15), 8596–8638.

Yu, P. (2005). Protein secondary structures (α-helix and β-sheet) at a cellular level and protein fractions in relation to rumen degradation behaviours of protein: a new approach. *British Journal of Nutrition*, *94*(5), 655–665.

Yu, X., Bals, O., Grimi, N., & Vorobiev, E. (2015). A new way for the oil plant biomass valorization: Polyphenols and proteins extraction from rapeseed stems and leaves assisted by pulsed electric fields. *Industrial Crops and Products, 74*, 309-318.

YuCetepe, A., SarOglu, O., Bildik, F., Ozcelik, B., & Daskaya-Dikmen, C. (2018). Optimisation of ultrasound-assisted extraction of protein from Spirulina platensis using RSM. *Czech Journal of Food Sciences, 36*(1), 98-108.

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*, *112*, 106330.

Yue, J., Zhu, Z., Yi, J., Li, H., Chen, B., & Rao, J. (2022). One-step extraction of oat protein by choline chloride-alcohol deep eutectic solvents: Role of chain length of dihydric alcohol. *Food Chemistry*, *376*, 131943.

Yue, Y., Huang, Q., Fu, Y., & Chang, J. (2020). A quick selection of natural deep eutectic solvents for the extraction of chlorogenic acid from herba artemisiae scopariae. *RSC Advances*, *10*(39), 23403–23409.

Zahn, S., Kirchner, B., & Mollenhauer, D. (2016). Charge Spreading in Deep Eutectic Solvents. *ChemPhysChem*, 17(21), 3354–3358.

Zainal-Abidin, M. H., Hayyan, M., Hayyan, A., & Jayakumar, N. S. (2017). New horizons in the extraction of bioactive compounds using deep eutectic solvents: A review. *Analytica Chimica Acta*, *979*, 1–23.

Zainan, N. H., Thiruvenkadam, S., Danquah, M. K., & Harun, R. (2020). Biochemical analysis and potential applications of aqueous and solid products generated from subcritical water extraction of microalgae *Chlorella pyrenoidosa* biomass. *Journal of Applied Phycology, 32*(1), 111-126.

Zander, N. E., Orlicki, J. A., Rawlett, A. M., & Beebe, T. P. (2012). Quantification of protein incorporated into electrospun polycaprolactone tissue engineering scaffolds. *ACS Applied Materials and Interfaces*, *4*(4), 2074–2081.

Zayas, J. F. (1997). Foaming Properties of Proteins. In *Functionality of Proteins in Food* (pp. 260–309). Springer- Verlag Berlin Heidelberg.

Zeng, Q., Wang, Y., Huang, Y., Ding, X., Chen, J., & Xu, K. (2014). Deep eutectic solvents as novel extraction media for protein partitioning. *Analyst, 139*(10), 2565-2573.

Zha, F., Rao, J., & Chen, B. (2021). Plant-based food hydrogels: Constitutive characteristics,

formation, and modulation. *Current Opinion in Colloid & Interface Science*, *56*, 101505.

Zhang, C., He, Z., Liu, J., Zhang, Y., Marcucci, M., Li, Y., & Lin, Z. (2025). Improving Spirulina protein purification by addressing polysaccharides and alcohol-soluble components interference in ultrafiltration. *Future Foods*, *11*, 100581.

Zhang, C., Sanders, J. P., Xiao, T. T., & Bruins, M. E. (2015a). How does alkali aid protein extraction in green tea leaf residue: A basis for integrated biorefinery of leaves. *PLoS One, 10*(7), e0133046.

Zhang, C. W., Xia, S. Q., & Ma, P. S. (2016). Facile pretreatment of lignocellulosic biomass using deep eutectic solvents. *Bioresource Technology*, *219*, 1–5.

Zhang, H., Wang, Y., Xu, K., Li, N., Wen, Q., Yang, Q., & Zhou, Y. (2016). Ternary and binary deep eutectic solvents as a novel extraction medium for protein partitioning. *Analytical Methods, 8*(46), 8196-8207.

Zhang, J., Wang, J., Li, M., Guo, S., & Lv, Y. (2022). Effects of heat treatment on protein molecular structure and in vitro digestion in whole soybeans with different moisture content. *Food Research International*, *155*, 111115.

Zhang, J., Wen, C., Zhang, H., Duan, Y., & Ma, H. (2020). Recent advances in the extraction of bioactive compounds with subcritical water: A review. *Trends in Food Science and Technology, 95*, 183-195.

Zhang, J., Wen, C., Zhang, H., Zandile, M., Luo, X., Duan, Y., & Ma, H. (2018a). Structure of the zein protein as treated with subcritical water. *International Journal of Food Properties, 21*(1), 128-138.

Zhang, L., Li, Q., Zhang, W., Bakalis, S., Luo, Y., & Lametsch, R. (2024). Different source of commercial soy protein isolates: Structural, compositional, and physicochemical characteristics in relation to protein functionalities. *Food Chemistry*, *433*, 137315.

Zhang, L., Li, Q., Zhang, W., & Lametsch, R. (2025). Gelation and emulsification profiles of different commercial soy protein isolates: A proteomic insight through globulin subunit compositions and oxidative modifications. *Food Hydrocolloids*, 111173.

Zhang, L., Wang, L.-J., Jiang, W., & Qian, J.-Y. (2017a). Effect of pulsed electric field on functional and structural properties of canola protein by pretreating seeds to elevate oil yield. *LWT- Food Science and Technology, 84*, 73-81.

Zhang, L., Zhang, M., Sun, X., Chen, F., & Wu, Q. (2021a). Effects of AOT reverse micelle extraction on structure and emulsifying properties of soybean protein. *Journal of the American Oil Chemists' Society, 98*(9), 923-932.

Zhang, Q.T., Tu, Z.C., Wang, H., Huang, X.Q., Fan, L.-L., Bao, Z.-Y., & Xiao, H. (2015b). Functional properties and structure changes of soybean protein isolate after subcritical water treatment. *Journal of Food Science and Technology, 52*(6), 3412-3421.

Zhang, Q., De Oliveira Vigier, K., Royer, S., & Jérôme, F. (2012). Deep eutectic solvents: syntheses, properties and applications. *Chemical Society Reviews*, *41*(21), 7108–7146.

Zhang, S., Huang, W., Roopesh, M. S., & Chen, L. (2022). Pre-treatment by combining atmospheric cold plasma and pH-shifting to prepare pea protein concentrate powders with improved gelling properties. *Food Research International*, *154*.

Zhang, S., Sun, L., Ju, H., Bao, Z., Zeng, X.-a., & Lin, S. (2021b). Research advances and application of pulsed electric field on proteins and peptides in food. *Food Research International, 139*, 109914.

Zhang, W., Huang, L., Chen, W., Wang, J., & Wang, S. (2021). Influence of ultrasound-assisted ionic liquid pretreatments on the functional properties of soy protein hydrolysates. *Ultrasonics Sonochemistry*, *73*, 105546.

Zhang, X., Hou, Y., Zhang, F., & Luo, G. (2017b). Protein extraction from grape seeds by reverse micelles: optimization of the forward extraction. *Open Access Library Journal, 4*(6), 1-12.

Zhang, X., Wang, Q., Liu, Z., Zhi, L., Jiao, B., Hu, H., Ma, X., Agyei, D., & Shi, A. (2023). Plant protein-based emulsifiers: Mechanisms, techniques for emulsification enhancement and applications. *Food Hydrocolloids*, *144*, 109008.

Zhang, Y., & Cremer, P. S. (2006). Interactions between macromolecules and ions: the Hofmeister series. *Current Opinion in Chemical Biology*, *10*(6), 658–663.

Zhang, Y., Sharan, S., Rinnan, Å., & Orlien, V. (2021). Survey on Methods for Investigating Protein Functionality and Related Molecular Characteristics. *Foods*, *10*(11), 2848.

Zhang, Z., Wang, Y., Dai, C., He, R., & Ma, H. (2018b). Alkali extraction of rice residue protein isolates: Effects of alkali treatment conditions on lysinoalanine formation and structural characterization of lysinoalanine-containing protein. *Food Chemistry, 261*, 176-183.

Zhao, C., Yin, H., Yan, J., Niu, X., Qi, B., & Liu, J. (2021). Structure and acid-induced gelation properties of soy protein isolate-maltodextrin glycation conjugates with ultrasonic pretreatment. *Food Hydrocolloids*, *112*, 106278.

Zhao, J., Liu, D., Chen, F., & Liu, G. (2012). Functional properties of sesame seed protein prepared by two different methods. *Journal of the Chemical Society of Pakistan, 34*(5), 1101-1106.

Zhao, R., Liu, X., Liu, W., Liu, Q., Zhang, L., & Hu, H. (2022). Effect of high-intensity ultrasound on the structural, rheological, emulsifying and gelling properties of insoluble potato protein isolates. *Ultrasonics Sonochemistry*, *85*, 105969.

Zhao, X., Liu, H., Zhang, X., & Zhu, H. (2019). Comparison of structures of walnut protein fractions obtained through reverse micelles and alkaline extraction with isoelectric precipitation. *International Journal of Biological Macromolecules, 125*, 1214-1220.

Zhao, X., Zhang, X., Liu, H., Zhang, G., & Ao, Q. (2018). Functional, nutritional and flavor characteristic of soybean proteins obtained through reverse micelles. *Food Hydrocolloids, 74*, 358-366.

Zhao, X., Zhu, H., Zhang, B., Chen, J., Ao, Q., & Wang, X. (2015). XRD, SEM, and XPS Analysis of Soybean Protein Powders Obtained Through Extraction Involving Reverse Micelles. *Journal of the American Oil Chemists' Society*, *92*(7), 975–983.

Zheng, L., Regenstein, J. M., Zhou, L., & Wang, Z. (2022). Soy protein isolates: A review of their composition, aggregation, and gelation. *Comprehensive Reviews in Food Science and Food Safety*, *21*(2), 1940–1957.

Zhou, F., Zhao, M., Su, G., Cui, C., & Sun, W. (2014). Gelation of salted myofibrillar protein under malondialdehyde-induced oxidative stress. *Food Hydrocolloids*, *40*, 153–162.

Zhou, M., Liu, J., Zhou, Y., Huang, X., Liu, F., Pan, S., & Hu, H. (2016). Effect of high intensity ultrasound on physicochemical and functional properties of soybean glycinin at different ionic strengths. *Innovative Food Science & Emerging Technologies*, *34*, 205–213.

Zhu, J., & Fu, Q. (2012). Optimization of ultrasound-assisted extraction process of perilla seed meal proteins. *Food Science and Biotechnology, 21*(6), 1701-1706.

Zhu, K.-X., Sun, X.-H., Chen, Z.-C., Peng, W., Qian, H.-F., & Zhou, H.-M. (2010). Comparison of functional properties and secondary structures of defatted wheat germ proteins separated by reverse micelles and alkaline extraction and isoelectric precipitation. *Food Chemistry, 123*(4), 1163-1169.

Zhu, S., Li, H., Zhu, W., Jiang, W., Wang, C., Wu, P., Zhang, Q., & Li, H. (2016). Vibrational analysis and formation mechanism of typical deep eutectic solvents: An experimental and theoretical study. *Journal of Molecular Graphics and Modelling*, *68*, 158–175.

Żmudziński, D., Goik, U., & Ptaszek, P. (2021). Functional and Rheological Properties of Vicia faba L. Protein Isolates. *Biomolecules*, *11*(2), 178.

Appendices

Appendix 1: Table 3.1S. ANOVA analysis in RSM model for protein extraction using DES from fava bean

Source	DF	SS	MS	F-Value	P-Value
Model	14	8061.41	575.82	77.25	<0.000
Linear					
X1	1	1385.59	1385.59	185.89	<0.000
X2	1	1240.53	1240.53	166.43	<0.000
X3	1	2001.46	2001.46	268.52	<0.000
X4	1	25.45	25.45	3.41	0.083
X5	1	4385.60	4385.60	588.37	<0.000
Square					
X1X1	1	39.14	39.14	5.25	0.036*
X2X2	1	100.66	100.66	13.50	0.002*
X3X3	1	129.02	129.02	17.31	0.001*
X4X4	1	47.29	47.29	6.35	0.023*
X5X5	1	223.92	223.92	30.04	<0.000
2-Way Interaction					
X1X5	1	551.52	551.52	73.99	<0.000
X2X3	1	172.30	172.30	23.12	<0.000
X2X4	1	83.83	83.83	11.25	0.004*
X3X4	1	52.60	52.60	7.06	0.017*
Error	16	119.26	7.45		
Lack-of-Fit	11	79.17	7.20	0.90	0.593
Pure Error	5	40.09	8.02		
Total	30	8180.67			
R^2 = 0.9854					
R^2 (adj) = 0.9727					

R^2(pred)= 0.9468

ANOVA analysis in RSM model for input variables; X_1 - ChCl : Gly molar ratio (w/w), X_2 - Solid: liquid ratio (w/w), X_3 - extraction temperature (°C), X_4-extraction time (hours) and X_5-water content (w/w). DF- Degree of freedom, SS- Sum of Square, MS- Mean square, R^2-coefficient of determination, R^2 (adj)- adjusted coefficient of determination, and R^2 (pred)- predicted coefficient of determination.

Appendix 2. Table 3.2S. Multiple response prediction and validation of the optimal protein extraction

Variables	Optimized values
ChCl: Glyc molar ratio (w/w)	1:2
Solid: liquid ratio (w/w)	1:28
Temperature (°C)	50C
Time (hours)	1hr
Water % (w/w)	40
Predicted protein content (mg/g DW)	**125.30**
95% confidence interval	(111.18,139.41)
Experimental values (mg/g DW)	**124.28 ± 0.91**

Optimized values obtained from the polynomial equation of RSM model with $P < 0.05$ and lack of fit $P > 0.05$. All data were analyzed by analysis of variance (n=3) and expressed as dry weight basis. ChCl- Choline chloride, Gly- Glycerol

Appendix 3. Table 3.3S. Protein extraction yield evaluated under optimum extraction conditions at different pH

pH	Mean extraction yield mg/g DW
5.0	122.62 ± 5.93[a]
5.5	120.87 ± 1.71[a]
6.0	121.25 ± 3.30[a]
7.0	121. 22 ± 4.48[a]
7.5	120.04 ± 4.35[a]
8.0	126.15 ± 3.46[a]

Protein yield obtained under different pH conditions using DES extraction from fava bean. All data were analyzed by analysis of variance followed by Tukey's test (n=3) and expressed as dry weight (DW) basis. Same letters within the same column indicated no significant difference (P>0.05).

Appendix 4. Table 3.4S. Physicochemical properties of prepared DES

DES Properties	Pure DES (100% w/w DES) Choline chloride: glycerol molar ratio of 1:2	60% (w/w) DES with 40% w/w water Choline chloride: glycerol molar ratio of 1:2
pH	5.84 ± 0.02^{a}	5.25 ± 0.02^{b}
Conductivity (mS/cm)	0.613 ± 0.02^{a}	17.38 ± 0.16^{b}
Viscosity (cP)	329.80 ± 1.85^{a}	9.43 ± 0.12^{b}
Density (g/mL)	1.20 ± 0.001^{a}	1.14 ± 0.003^{b}

Physicochemical properties of 100% pure deep eutectic solvent (DES) and DES containing 40% (w/w) water with 1:2 molar ratio of choline chloride and glycerol. The pH, conductivity and viscosity were measured at 25 °C and density was measured at ambient temperature (23 °C). All data were analyzed by analysis of variance followed by Tukey's test (n=3). Different letters within the same raw indicated significant difference ($P > 0.05$).

Appendix 5. Table 4.1S. Protein composition of extracted protein isolates (Protein molecular band %)

Protein subunit	DESE-FBPI %	ALKE-FBPI %	SSE-FBPI %	CS-PI %
Non-Reducing condition				
Convicilin (63-73 kDa)	11.49	9.95	8.82	-
Vicilin (37-43 kDa)	8.38	5.92	9.17	-
Legumin (42-51 kDa)	17.64	17.64	16.45	-
Soy protein 11S (kDa)	-	-	-	12.2
Soy protein 7S (kDa)	-	-	-	11.19
Reducing Condition				
Convicilin (62-65 kDa)	5.55	6.16	5.67	-
Vicilin (43-45 kDa)	9.92	10.64	10.41	-
Legumin α (29-34 kDa)	21.76	21.25	24.26	-
Legumin β (17-21 kDa)	20.26	20.53	21.95	-
11S/7S	**2.72**	**2.48**	**2.87**	**-**
Soy protein 11S (kDa)	-	-	-	38.66
Soy protein 7S (kDa)	-	-	-	23.24
11S/7S	**-**	**-**	**-**	**1.66**

The 11S/7S ratios were calculated based on the sum of the relative percentages of 11S (α-legumin + β-legumin) and 7S (convicilin + vicilin) proteins. DES (DESE-FBPI), alkaline (ALKE-FBPI), and salt-extracted (SSE-FBPI) fava bean protein isolates and commercial soy protein isolates (CS-PI).

Appendix 6. Table 5.1S. Multiple response prediction and validation of optimized variables for UP-assisted DES protein extraction

Variable	**Optimized values**			
Amplitude %	61.41 (~62)			
Time (min)	6.9 (~7)			
Response	Fit	SE Fit	95% CI	95% PI
Predicted protein yield % (DW)	68.77	0.256	(68.168, 69.377)	(67.270, 70.275)
Experimented protein yield % (DW)	68.48 ± 1.38			

Optimized values obtained from the polynomial equation of RSM model with $P < 0.05$ and lack of fit $P > 0.05$. All data were analyzed by analysis of variance (n=3) and expressed as dry weight basis. DES- deep eutectic solvent containing choline chloride and glycerol.

Appendix 7. Table 5.2S. ANOVA analysis in RSM model for protein extraction using UP-assisted DES protein extraction from fava bean

Analysis of Variance (ANOVA)					
Source	DF	Adj SS	Adj MS	F-Value	P-Value
Model	**5**	**62.671**	**12.5342**	**37.02**	**0.000**
Linear	**2**	**18.0924**	**9.0462**	**26.72**	**0.001**
Amplitude % ***(X1)***	**1**	**0.0986**	**0.0986**	**0.29**	**0.606**
Time (min) ***(X2)***	**1**	**17.9938**	**17.9938**	**53.15**	**0.000**
Square	**2**	**42.7016**	**21.3508**	**63.07**	**0.000**
*X1*X1*	1	2.1524	2.1524	6.36	0.04
*X2*X2*	1	42.3123	42.3123	124.98	0.000
2-Way Interaction	**1**	**1.8769**	**1.8769**	**5.54**	**0.051**
*X1*X2*	1	1.8769	1.8769	5.54	0.051
Error	7	2.3698	0.3385		
Lack-of-Fit	**3**	**1.8234**	**0.6078**	**4.45**	**0.092**
Pure Error	4	0.5464	0.1366		
Total	12	65.0408			
$\boldsymbol{R^2 = 0.9636}$					

ANOVA analysis in RSM model with $P < 0.05$ and lack of fit $P > 0.05$ for input variables; X_1 – Amplitude%, X_2 – Time (min). DF - Degree of freedom, SS - Sum of squares, MS - Mean square, R^2–Coefficient of determination.

Appendix 8. Table 5.3S. Independent variables used in experimental design (CCD) for EP-assisted DES protein extraction

Input Variables	Levels				
	-1.68	-1	0	1	1.68
Enzyme concentration (FBG/10g) (*X1*)	6	11	18	25	30
Temperature (°C) (*X2*)	30	34	40	46	50
pH (*X3*)	3	3.41	4	4.56	5

CCD- central composite design in Response Surface Methodology (RSM), FBG-Fungal beta-glucanase /10 g of fava bean flour as substrate

Appendix 9. Table 5.4S. Central composite design (CCD) matrix and response variable (protein yield%) predicted from EP-assisted DES protein extraction

Run	Independent variables [a]			Protein Yield % (DW) [b]
	X1	**X2**	**X3**	
1	11	34	3.41	31.27
2	18	40	4.00	30.21
3	18	40	4.00	29.53
4	25	46	3.41	14.32
5	11	46	4.59	35.58
6	25	46	4.59	34.35
7	18	40	4.00	31.70
8	18	30	4.00	31.92
9	18	40	5.00	42.58
10	25	34	3.41	24.70
11	6	40	4.00	32.45
12	11	34	4.59	40.52
13	18	50	4.00	23.36
14	18	40	4.00	28.80
15	18	40	4.00	26.59
16	25	34	4.59	36.90
17	18	40	4.00	31.71
18	11	46	3.41	21.79

19	30	40	4.00	29.57
20	18	40	3.00	22.36

[a] Coded symbols and levels of independent variables refer to Table 1. [b] Averages of Triplicated determination (n=3) predicted protein yield % on a dry weight basis (DW), *X1*- Enzyme concentration *i.e*. Fungal beta-glucanase/10 g of fava bean flour as substrate, *X2*- Temperature (°C), *X3*- pH

Appendix 10. **Table 5.5S**. ANOVA analysis in RSM model for protein extraction using EP-assisted DES protein extraction from fava bean

Analysis of Variance					
Source	DF	Adj SS	Adj MS	F-Value	P-Value
Model	**7**	**805.5**	**115.071**	**38.86**	**0.000**
Linear	**3**	**752.495**	**250.832**	**84.7**	**0.000**
X1	1	41.28	41.28	13.94	0.003
X2	1	127.582	127.582	43.08	0.000
X3	1	583.632	583.632	197.08	0.000
Square	**2**	**23.298**	**11.649**	**3.93**	**0.049**
X2*X2	1	11.339	11.339	3.83	0.074
X3*X3	1	9.859	9.859	3.33	0.093
2-Way Interaction	**2**	**29.708**	**14.854**	**5.02**	**0.026**
X1*X3	1	10.564	10.564	3.57	0.083
X2*X3	1	19.143	19.143	6.46	0.026
Error	12	35.537	2.961		
Lack-of-Fit	**7**	**16.732**	**2.39**	**0.64**	**0.718**
Pure Error	5	18.806	3.761		
Total	19	841.037			
$\boldsymbol{R^2}$**=0.9577**					

ANOVA analysis in RSM model with $P < 0.05$ and lack of fit $P > 0.05$ for input variables; *X1*-Enzyme concentration, *i.e*., Fungal beta-glucanase/10 g of fava bean flour as substrate, *X2*-Temperature (°C), *X3*-pH, DF-Degree of freedom, SS-Sum of Squares, MS-Mean square, R^2 – coefficient of determination.

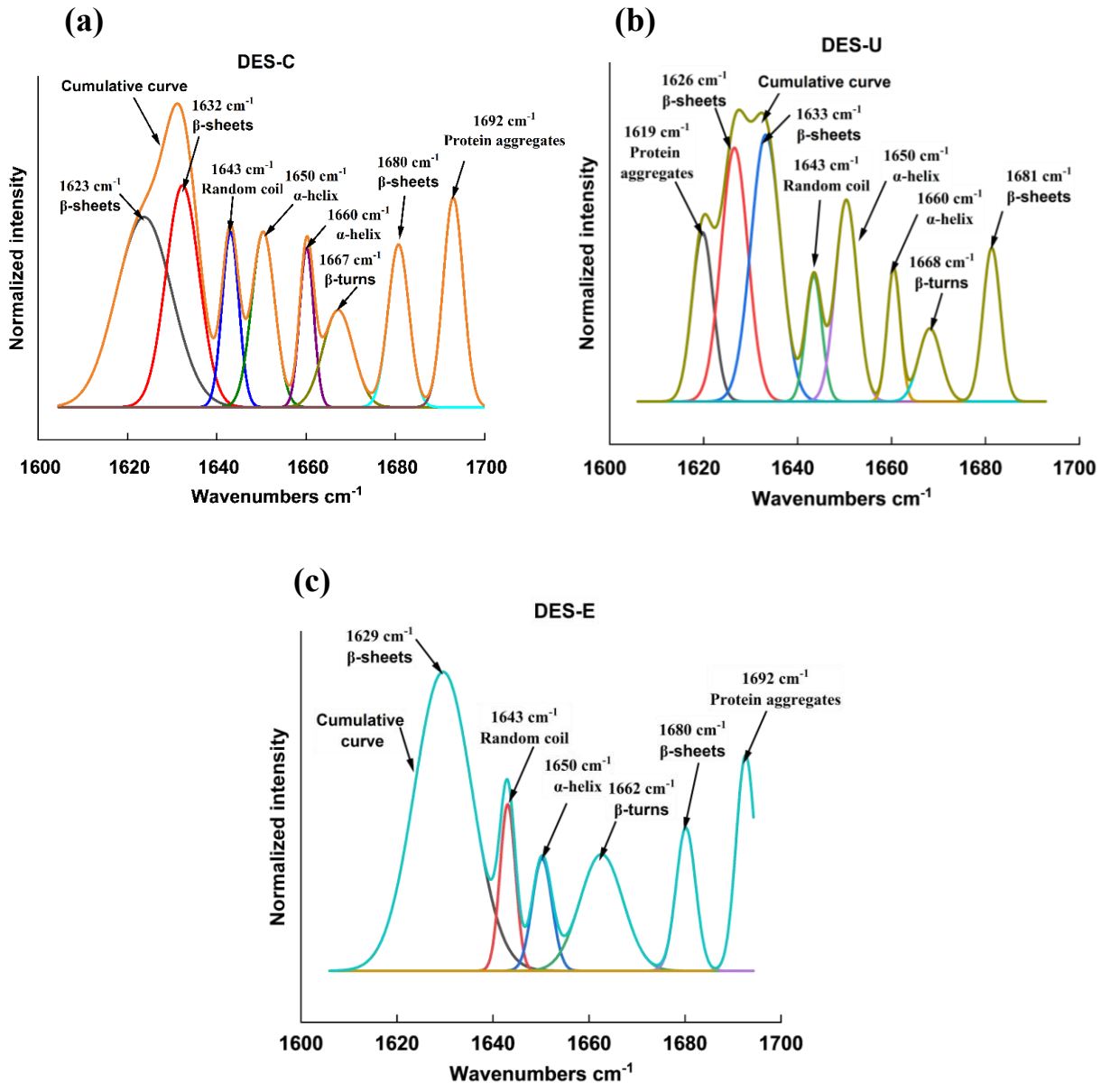
Appendix 11. Table 5.6S. Multiple response prediction of optimized variables for EP- assisted DES protein extraction

Variable	**Optimized values**			
Enzyme concentration (FBG/10g)	30			
Temperature (°C)	38.48 (~38.5)			
pH	5.0			
Response	Fit	SE Fit	95% CI	95% PI
Predicted protein yield % (DW)	43.76	2.32	38.70-48.81	37.47-50.05
Experimented protein yield % (DW)	45.36	-	-	-

Optimized values obtained from the polynomial equation of RSM model with $P < 0.05$ and lack of fit $P > 0.05$. All data were analyzed by analysis of variance (n=3) and expressed as dry weight basis. DES- deep eutectic solvent containing choline chloride and glycerol.

Appendix 12. Figure 5.1S: Color visualization of fava bean protein isolates extracted by various pre-treatment methods, *i.e.,* DES-C (control sample without pre-treatment), DES-E (enzymatic-assisted DES extraction), and DES-U (ultrasonication-assisted DES extraction).

Appendix 13. Figure 5.2S: FTIR spectra for the fava bean protein isolates extracted by various pre-treatment methods, *i.e.,* (a) control sample without pre-treatment (DES-C), (b) ultrasonication-assisted DES extraction (DES-U), and (c) enzymatic-assisted DES extraction (DES-E).

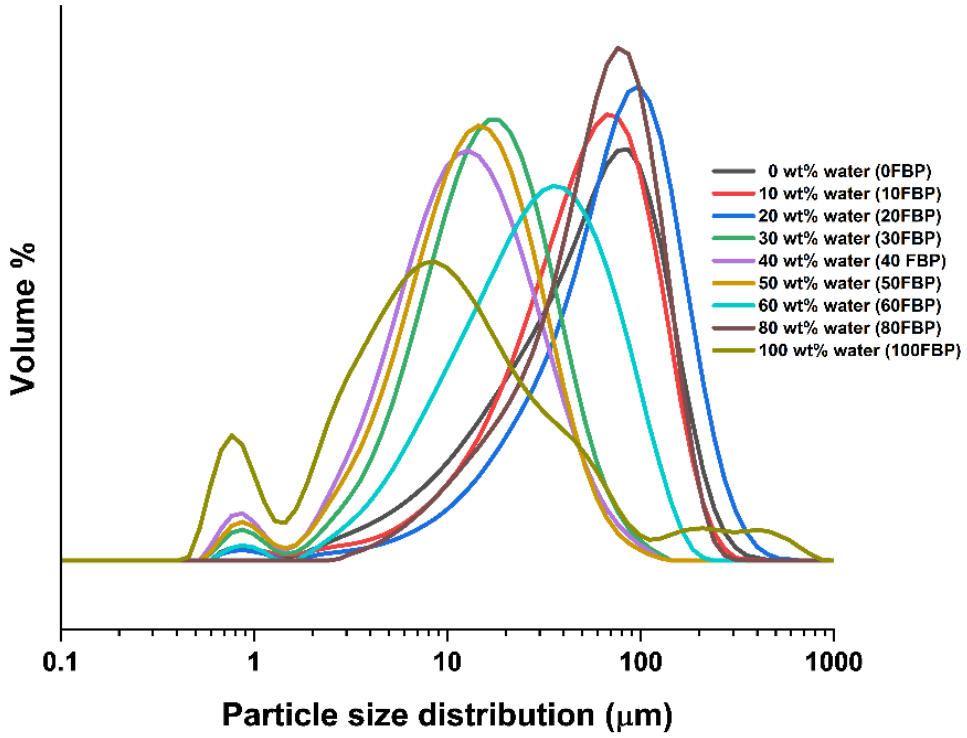


Appendix 14. Table 6.1S. Physical properties of ChCl/Gly DES system (1:2) at different water substitution levels

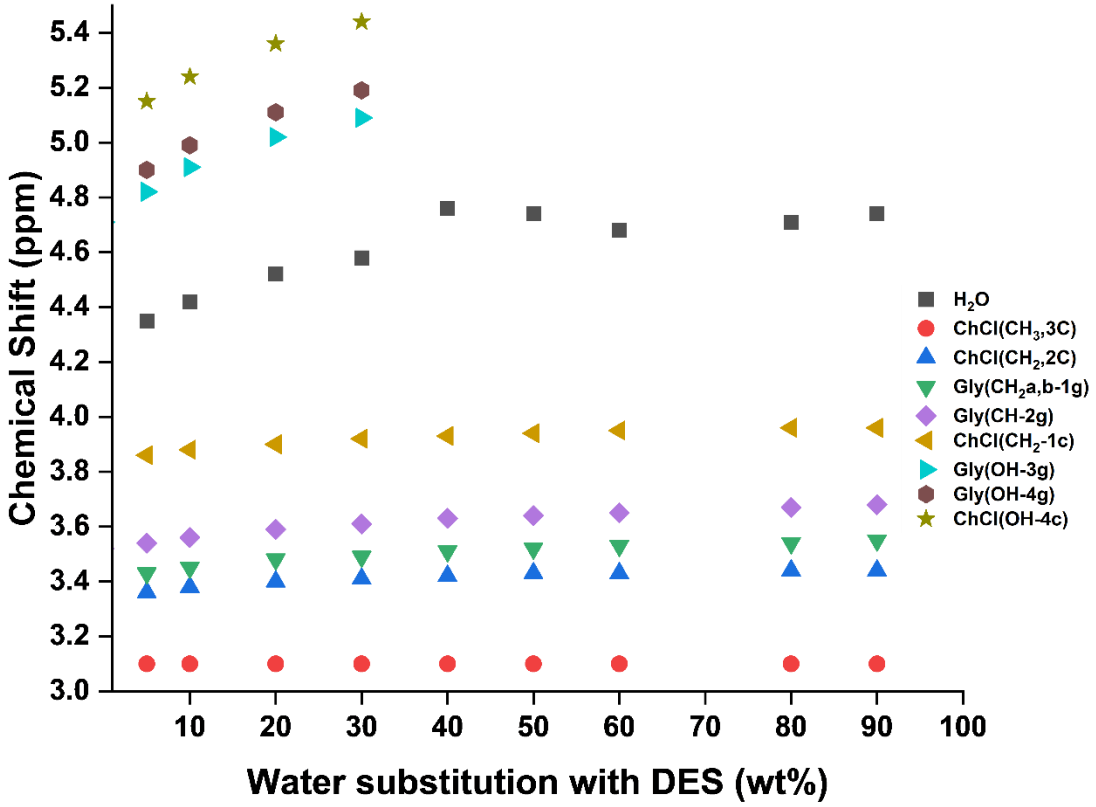
Water substitution with DES (wt%)	Density (mg/ml)	Ionic conductivity (mS/m)	pH	Viscosity (Pa.s)
0	1.206 ±0.00^{a}	1.173 ± 0.05^{h}	5.727 ± 0.18^{a}	0.314 ± 0.01^{a}
10	1.185 ± 0.00^{b}	4.628 ± 0.05^{g}	5.426 ± 0.11ab^{c}	0.069 ± 0.00^{b}
20	1.167 ± 0.00^{c}	10.340 ± 0.18^{f}	4.783 ± 0.24d^{e}	0.026 ± 0.00^{c}
30	1.146 ± 0.00^{d}	18.713 ± 0.57^{e}	4.926 ± 0.01^{cd}	0.011 ± 0.00^{cd}
40	1.124 ± 0.00^{e}	27.903 ± 0.37^{c}	4.260 ± 0.39^{e}	0.006 ± 0.00^{d}
50	1.103 ± 0.00^{f}	34.986 ± 0.09^{b}	5.136 ± 0.11^{bcd}	0.003 ± 0.00^{d}
60	1.094 ± 0.00^{f}	39.113 ± 0.25^{a}	5.260 ± 0.13^{abcd}	0.002 ± 0.00^{d}
80	1.043 ± 0.00^{g}	33.943 ± 0.22^{b}	5.606 ± 0.04^{ab}	0.001 ± 0.00^{d}

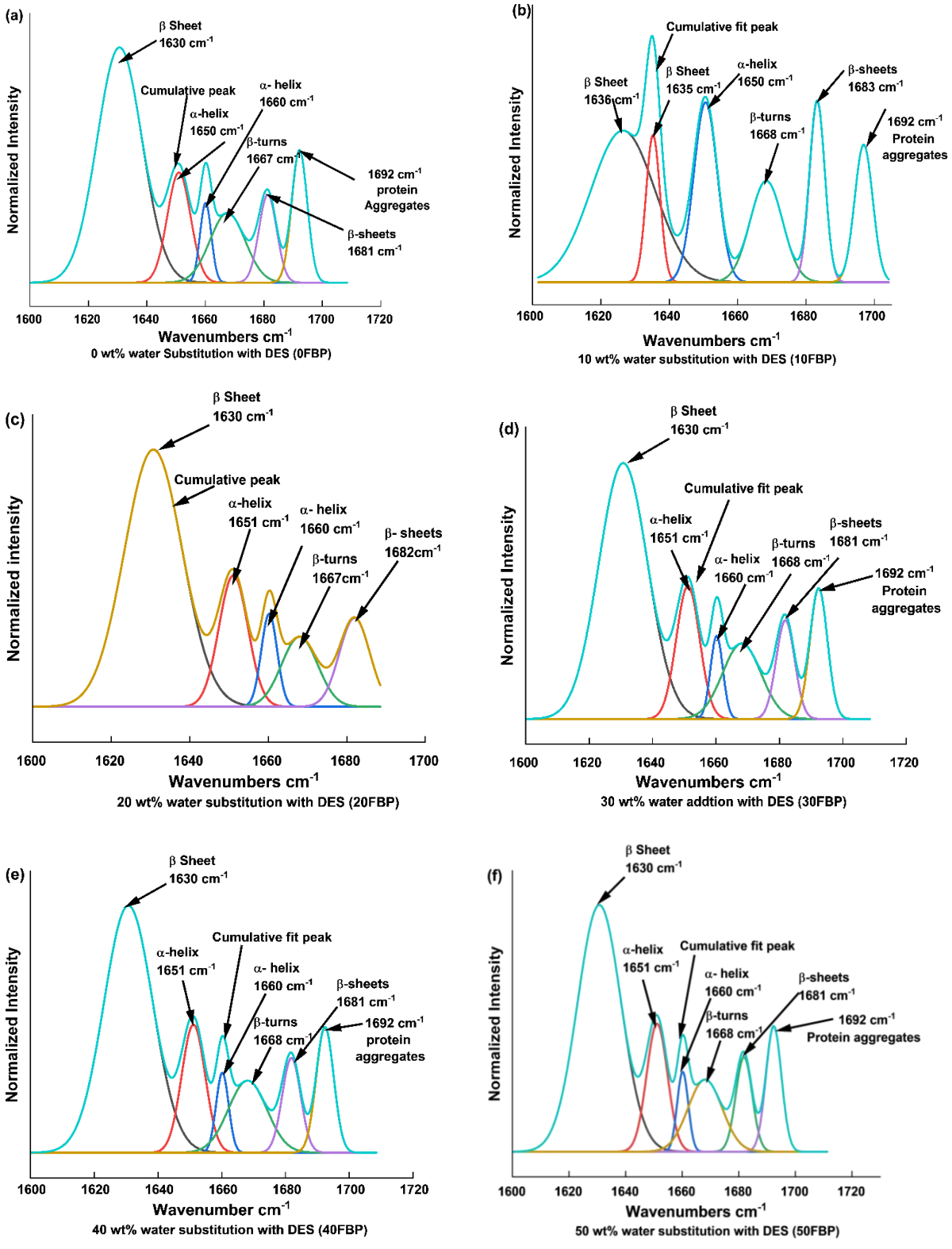
All data were analyzed by Analysis of Variance (ANOVA) followed by Tukey's test (n=3). Different superscripts within a column indicate a significant difference ($P < 0.05$).

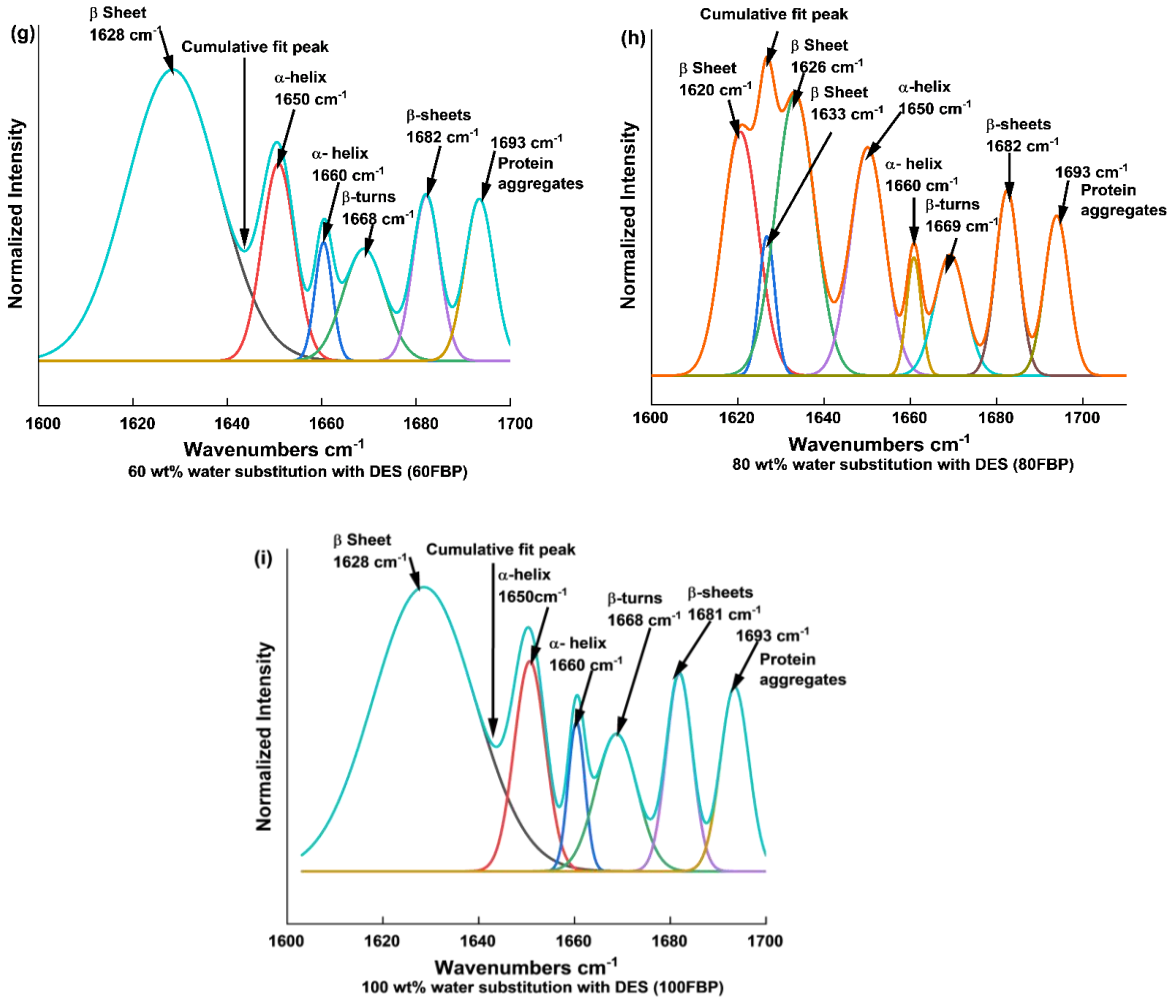
Appendix 15. Figure 6.1S. Particle size distribution of fava bean protein (FBP) extracted using DES with varying water substitution levels (0-100 wt%).



Appendix 16. Figure 6.2S. ^{1}H NMR Chemical shift deviation of choline chloride: glycerol (1:2) DES system as a function of water content. ChCl- choline chloride, Gly- Glycerol




(a)
β Sheet
1630 cm-1
Cumulative peak
α- helix
1660 cm-1
α-helix
1650 cm-1
β-turns
1667 cm-1
1692 cm-1
protein
Aggregates
β-sheets
1681 cm-1
Normalized Intensity
1600 1620 1640 1660 1680 1700 1720
Wavenumbers cm-1
0 wt% water Substitution with DES (0FBP)
(b)
Cumulative fit peak
β Sheet
1636 cm-1
β Sheet
1635 cm-1
α-helix
1650 cm-1
β-sheets
1683 cm-1
β-turns
1668 cm-1
1692 cm-1
Protein
aggregates
Normalized Intensity
1600 1620 1640 1660 1680 1700
Wavenumbers cm-1
10 wt% water substitution with DES (10FBP)
(c)
β Sheet
1630 cm-1
Cumulative peak
α-helix
1651 cm-1
α- helix
1660 cm-1
β- sheets
1682cm-1
β-turns
1667cm-1
Normalized intensity
1600 1620 1640 1660 1680 1700
Wavenumbers cm-1
20 wt% water substitution with DES (20FBP)
(d)
β Sheet
1630 cm-1
Cumulative fit peak
α-helix
1651 cm-1
β-sheets
1681 cm-1
β-turns
1668 cm-1
α- helix
1660 cm-1
1692 cm-1
Protein
aggregates
Normalized Intensity
1600 1620 1640 1660 1680 1700 1720
Wavenumbers cm-1
30 wt% water addtion with DES (30FBP)
(e)
β Sheet
1630 cm-1
Cumulative fit peak
α-helix
1651 cm-1
α- helix
1660 cm-1
β-sheets
1681 cm-1
β-turns
1668 cm-1
1692 cm-1
protein
aggregates
Normalized Intensity
1600 1620 1640 1660 1680 1700 1720
Wavenumber cm-1
40 wt% water substitution with DES (40FBP)
(f)
β Sheet
1630 cm-1
α-helix
1651 cm-1
Cumulative fit peak
α- helix
1660 cm-1
β-sheets
1681 cm-1
β-turns
1668 cm-1
1692 cm-1
Protein aggregates
Normalized Intensity
1600 1620 1640 1660 1680 1700 1720
Wavenumbers cm-1
50 wt% water substitution with DES (50FBP)



Appendix 17. Figure 6.3S. (A-I) Peak fitting of amide I with the relative proportion of each secondary structure of DES-extracted fava bean proteins at (a) 0% wt water (b) 10 wt% water (c) 20 wt% (d) 30 wt%, (e) 40 wt%, (f) 50 wt%, (g) 60 wt%, (h) 80 wt% and (i) 100 wt% water levels.

Appendix 18. 7.1S DES extraction

DES protein extraction was carried out by using optimized conditions developed in our previous study (Hewage et al., 2024) with slight modifications. First, fava bean flour (FBF) was mixed with 60% DES containing ChCl and Gly (1:2 molar ratio, w/w) and 40% water (w/w) at a 12.5:1 liquid: solid ratio. It was then homogenized at a speed of 20,000 rpm for 2 min (Model 850, Fisher Scientific, Ottawa, ON, Canada), followed by protein extraction using a circulating water bath (Model 6200 H7, Fisher Scientific Inc, Pittsburgh, PA, USA) at 200 rpm, 50 °C for 1 hrs. The supernatant collected after centrifugation (1507 g, 10 min, 4 °C) was dialyzed (3.5 kDa) at 4 °C for 5 days, with continuous refreshment with distilled water. Finally, the resulting protein solution was freeze-dried and stored at -20 °C.

Appendix 19. **7.2S** Alkaline extraction (AE)

Alkaline extraction followed by isoelectric precipitations was performed based on the method described previously (Karaca et al., 2011) with slight modifications. FBF was dispersed in a 1:10 (w/v) solid: liquid ratio and homogenized (Model 850, Fisher Scientific, Ottawa, ON, Canada) at 14,000 rpm for 2 min. The pH of the slurry was then adjusted to 9.5 using 5 M NaOH. After 2 hrs of stirring (200 rpm) at 22 ºC, the mixture was centrifuged at 17,709 g, 4 ºC for 20 min. The supernatant was adjusted to pH 4.5 using 1 M HCl to precipitate the proteins. Thereafter, precipitated proteins were collected using centrifugation as above, and protein pellets were dispersed in distilled water. Before freeze-drying, the protein slurry was adjusted to a pH of 7.0 using 1 M NaOH, and the dried protein was stored at -20 °C for further use.

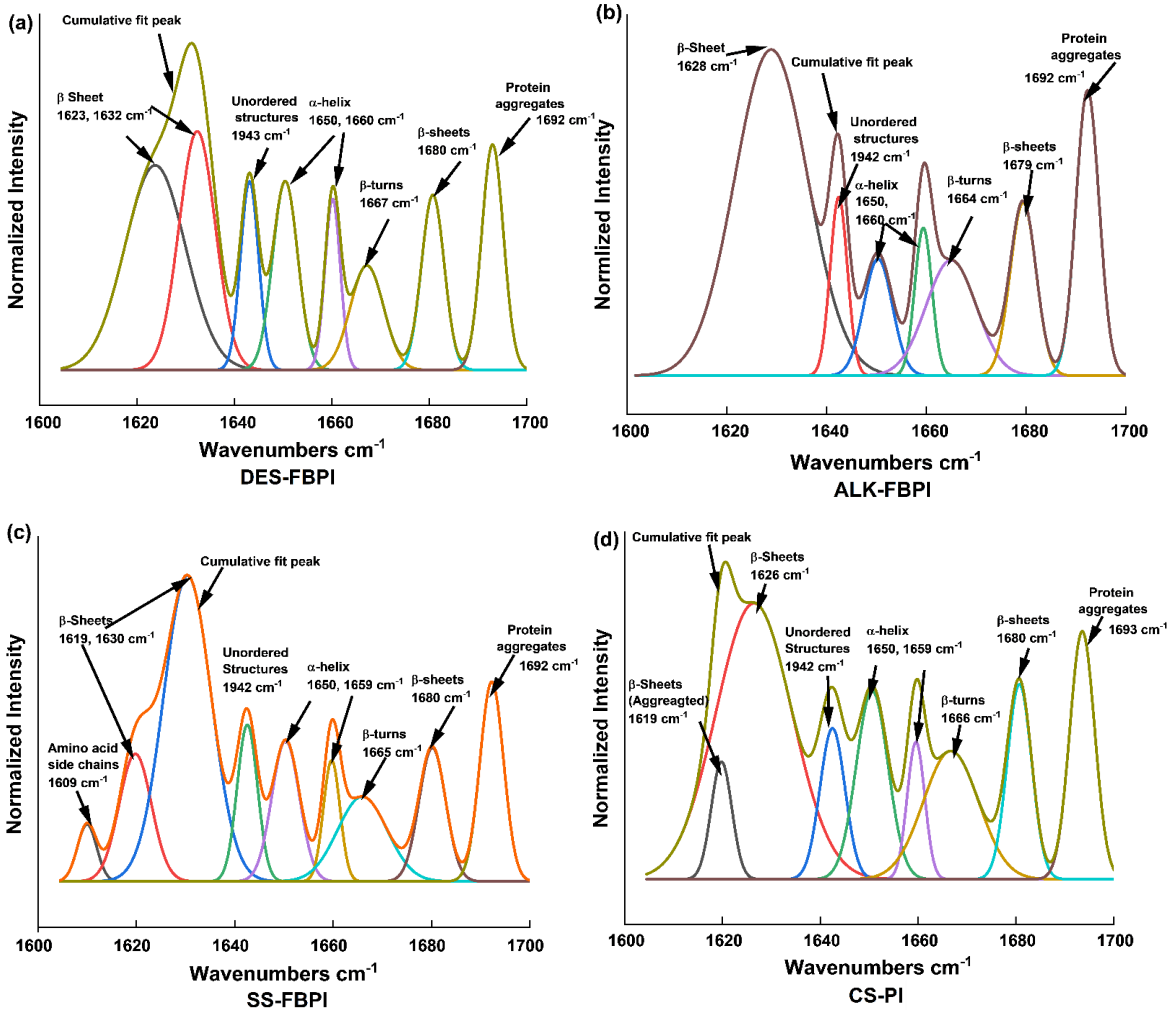
Appendix 20. 7.3S Salt extraction (SE)

Salt-extracted fava bean protein isolates were obtained according to the protocol published previously (Karaca et al., 2011) with slight modifications. Briefly, FBF was mixed with a 5% aqueous solution of sodium sulfate at a 1:10 (w/v) ratio and homogenized (Model 850, Fisher Scientific, Ottawa, ON, Canada) at 14,000 rpm for 2 min. The slurry was then adjusted to a pH of 7.0 with 0.1 M NaOH and stirred for 2 hrs at 200 rpm and 22 °C. The mixture was centrifuged at 17,709 g for 20 min at 4 °C, and the supernatant was dialyzed using 3.5 kDa MWCO tubing at 4 °C for 72 hrs, with water changes made daily. The dialyzed solution was then freeze-dried and stored at -20 °C until further analysis.

Appendix 21. 7.4S. Gel preparation and determination of Least Gel concentration (LGC)

LGC of protein fractions was determined using the method adopted by Vogelsang-O'Dwyer et al. (2020) with modifications. Proteins were dispersed in 0.1 M acetate (pH 3.0 and 5.0), phosphate (pH 7.0), and Tris-HCl (pH 9.0) buffers at concentrations ranging from 5% to 20% (based on protein weight/volume) and hydrated at 4 °C for 16 hrs. Prepared samples were then heated at 90 °C for 1 hr, cooled immediately using running water, and stored at 4 °C overnight. The LGC was defined as the protein dispersions that did not slip down when the tubes were inverted. The gels formed were categorized as firm (F) when the gel could be taken out from the tube without breaking, whereas soft gels (S) when they fell apart.

Appendix 22. Figure 7.1S. Peak fitting of Amide I peak with the relative proportion of each secondary structure of proteins extracted by various methods, *i.e.,* (A) Deep eutectic solvent-extracted fava bean protein isolates (DES-FBPI), (B) Alkaline-extracted fava bean protein isolates (ALK-FBPI), (C) Salt-extracted fava bean protein isolates (SS-FBPI), and (D) Commercial soy protein isolates (CS-PI).



Appendix 23. Table 7.1S: Least gel concentration (LGC) of extracted protein isolates

pH	3						5						7						9					
Ingredients	5 %	8 %	10 %	12 %	15 %	20 %	5 %	8 %	10 %	12 %	15 %	20 %	5 %	8 %	10 %	12 %	15 %	20 %	5 %	8 %	10 %	12 %	15 %	20 %
DES-FBPI	S	S	S	S	S	S	×	×	S	S	S	S	×	×	S	S	S	F	×	×	×	S	S	S
ALK-FBPI	S	S	S	S	S	S	S	S	S	S	S	S	×	×	×	S	S	S	×	×	×	S	S	S
SS-FBPI	×	S	S	S	S	S	×	S	S	S	S	S	×	×	S	S	S	S	×	×	×	S	S	S
CS-PI	×	S	S	S	S	S	×	S	S	S	S	S	S	S	S	F	F	F	×	×	×	S	F	F

Least gel concentration evaluated by protein concentration from 5-20% w/v at various pH ranges, 3.0-9.0 of protein isolates obtained by various extraction methods, *i.e.,* Deep eutectic solvent-extracted fava bean protein isolates (DES-FBPI), Alkaline-extracted fava bean protein isolates (ALK-FBPI), salt-extracted fava bean protein isolates (SS-FBPI), and commercial soy protein isolates (CS-PI). S- soft gels- gels easily break when handling, F- firm gels- strong gels that hold their shape when handling, ×- no gel formation.

Appendix 24. Table 7.2S. Intermolecular interactions, gel hardness and water holding capacity of gels prepared from protein isolates

Parameters	Protein Gels			
	DES-FBG	**ALK-FBG**	**SS-FBG**	**CSG**
Gel molecular interaction [soluble protein (mg/ml)]				
Ionic bonds	1.01±0.09^{b}	0.89±0.05^{b}	2.35±0.46^{a}	0.15±0.04^{c}
Hydrogen bonds	0.60±0.42^{ab}	1.01±0.12^{a}	0.3±0.03^{b}	0.07±0.02^{b}
Hydrophobic interactions	5.44±0.39^{b}	4.67±0.30^{b}	2.62±0.32^{c}	8.90±0.08^{a}
Disulfide bonds	2.67±0.60^{a}	1.35±0.08^{bc}	1.69±0.57^{ab}	0.32±0.02^{c}
Gel hardness (N)	921.58±49.43^{a}	NP	NP	567.61±89.04^{b}
Water holding capacity of gels (%)	81.82±1.58^{c}	95.70±1.62^{b}	75.45±0.73^{d}	101.21±2.36^{a}

Intermolecular interactions, gel hardness and water holding capacity of gels prepared from protein isolates extracted by various methods *i.e.* Deep-eutectic solvent extracted fava bean protein isolates gels (DES-FBG), Alkaline-extracted fava bean protein isolates gels (ALK-FBG), salt-extracted fava bean protein isolates gels (SS-FBG), and commercial soy protein isolates gels (CSG). All data are presented as means and standard deviation were analyzed by analysis of variance (ANOVA) followed by Tukey's test (n=3). Different letters in each raw indicate a significant difference ($P < 0.05$). NP- Not performed.

Appendix 25. Table 7.3S. Amino acid composition of protein isolates

AA composition (mg/g protein)	Requirement pattern[1](mg/g protein)	DES-FBPI	ALK-FBPI	SS-FBPI	CS-PI
Essential (EAA)					
Histidine (His)	19	28.8	28.3	29.5	28.4
Isoleucine (Ile)	28	46.8	46.9	46.3	46.7
Leucine (Leu)	66	81	83.3	79.6	78.9
Lysine (Lys)	58	68	66.9	69.8	63.5
Threonine (Thr)	34	36.8	35	37	36.7
Tryptophan (Trp)	11	9.6	9.4	8.8	13.9
Valine (Val)	35	49.8	49.7	48.9	47.5
Total sulfur amino acids (Met + Cys)	25	**22.5**	**19.4**	**23.1**	**26.3**
Total aromatic amino acids (Phe + Tyr)	63	82.8	81	80.5	86.5
Non-essential (NEAA)					
Alanine (Ala)	-	43	41.6	43.2	43.1
Arginine (Arg)	-	87.1	89.8	86.4	71
Aspartic acid (Asp)	-	120.6	120.7	121	120.2
Glutamic acid (Glu)	-	184.8	189.8	186.6	198.5
Glycine (Gly)	-	41	40.4	41.3	39.2

Proline (Pro)	-	44.8	44.7	45.3	49.5
Serine (Ser)	-	52.5	53	52.6	50.5
Total EAA (mg/g protein)	-	**426**	**419.9**	**423.6**	**428.5**
Total NEAA (mg/g protein)	-	574	580.1	576.4	571.5
Total AA (g/100g sample)	-	86.4	89.7	87.1	86
First limiting amino acid	-	**Tryptophan**	**Met+Cys**	**Tryptophan**	**No deficiency**
Total hydrophilic AA		**578.6**	**583.5**	**582.9**	**568.8**
Total hydrophobic AA		**421.3**	**416.4**	**417**	**431.6**
hydrophilic: hydrophobic AA ratio		**1.37**	**1.40**	**1.40**	**1.32**

[1] FAO/WHO (1991) Expert Consultation Report for amino acid scoring patterns for pre-school children (2-5 years old). Amino acid composition of proteins extracted by various extraction methods, *i.e.,* Deep eutectic solvent-extracted fava bean protein isolates (DES-FBPI), Alkaline-extracted fava bean protein isolates (ALK-FBPI), salt-extracted fava bean protein isolates (SS-FBPI), and commercial soy protein isolates (CS-PI).

Appendix 26. Table 7.4S. Elemental composition of the surface layer of extracted protein isolates

Protein ingredients	Atomic %				N/C	O/C
	C 1s	**N 1s**	**O 1s**	**S 2p**		
DES-FBPI	78.81	7.21	13.73	0.25	0.09	0.17
ALK-FBPI	75.45	8.71	15.84	0	0.12	0.21
SS-FBPI	75.80	8.65	15.27	0.29	0.11	0.20
CS-PI	78.88	5.55	15.49	0.13	0.07	0.20

Elemental composition (C- carbon, N-Nitrogen, O-oxygen and S- sulfur) of protein extracted by various methods, *i.e.,* Deep eutectic solvent-extracted fava bean protein isolates (DES-FBPI), alkaline-extracted fava bean protein isolates (ALK-FBPI), salt-extracted fava bean protein isolates (SS-FBPI), and commercial soy protein isolates (CS-PI).

Appendix 27. Table 7.5S. XPS C1s, O1s, and N1s binding energies, area, and functional groups of protein isolates

Protein isolates	Peak components	Binding energy (eV)	Functional groups	Binding area
DES-FBPI		284.5	C-C/C-H	7133.3
	C1s	285.8	H-C-H	1477.7
		287.68	C=O	2007.3
		399.23	N-H/C-N	1037.7
	N1s	398.98	N-H/C-N	413.6
		399.53	C=N/C-N	262.9
		530.25	O-C=O	4636
	O1s	531.1	O-C=O/O=C-N	499.1
		532.51	C-O-C/C-OH	1062.7
ALK-FBPI		284.57	C-C/C-H	5535.8
	C1s	285.52	H-C-H	2901
		287.88	C=O	830.8

		398.89	N-H/C-N	1282.1
	N1s	399.01	N-H/C-N	515.5
		399.61	C=N/C-N	217.9
		530.78	O-C=O	4187.8
		531.87	O-C=O/O=C-N	1166.9
	O1s	532.68	C-O-C/C-OH	1038.7
SS-FBPI				
		284.44	C-C/C-H	5104.1
		285.75	H-C-H	1660
	C1s	287.66	C=O	1483.9
		398.96	N-H/C-N	841.9
	N1s	399.11	N-H/C-N	572.6
		400.1	C=N/C-N	98.61
		530.72	O-C=O	2674.8
	O1s	532	C-O-C/C-OH	2540.5
CS-PI		284.51	C-C/C-H	8092.6
	C1s	285.85	H-C-H	2146

	287.76	C=O	1407.2
N1s	398.93	N-H/C-N	956.1
	398.99	N-H/C-N	230.8
	399.61	C=N/C-N	214.5
O1s	530.15	O-C=O	5274.8
	531.37	O-C=O/O=C-N	2053.7

The binding energy of each spectrum of C 1s, N 1s, and O 1s of protein extracted by various methods, *i.e.,* Deep eutectic solvent-extracted fava bean protein isolates (DES-FBPI), alkaline-extracted fava bean protein isolates (ALK-FBPI), salt-extracted fava bean protein isolates (SS-FBPI), and commercial soy protein isolates (CS-PI). Functional groups were determined based on the literature; Guerrero et al., (2013), Zhao et al. (2015) and Zander et al. (2012).

Appendix 28. Figure 7.2S. Changes in the Tan (δ) during the temperature sweep of protein dispersions (pH-7.0, protein concentration 20% w/v) from protein isolates obtained from various extraction methods, *i.e.,* Deep eutectic solvent-extracted fava bean protein isolates (DES-FBPI), Alkaline-extracted fava bean protein isolates (ALK-FBPI), Salt-extracted fava bean protein isolates (SS-FBPI), and Commercial soy protein isolates (CS-PI).