

Chickpea as an Alternative Protein Source: Sustainable Processing Technologies for Chickpea Production

By

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Abstract

The global shift toward sustainable and health-conscious food systems has intensified interest in plant-based proteins, with chickpea emerging as a promising alternative due to its high protein content, functional versatility, and low environmental footprint. However, conventional protein extraction methods, including alkaline and salt-based techniques, are often limited by protein denaturation, reduced functionality, and high wastewater generation. This thesis investigates Deep Eutectic Solvents (DES) and Plasma-Activated Water (PAW) as innovative and environmentally sustainable processing strategies to enhance the yield, structural integrity, and functional performance of chickpea protein isolates. In the first study, a choline chloride – glycerol based DES system was optimized using a multi-level factorial design, evaluating liquid to solid ratio (5:1 – 25:1) and water substitution levels (40-80%). Optimal extraction conditions (15:1 liquid to solid ratio and 70% water substitution) produced significantly higher protein content (87.77%), yield (51.25%), and recovery rate (12.77%) compared to alkaline and monovalent salt extraction methods. DES-extracted CPI exhibited enhanced solubility across pH 3.0-9.0, superior emulsifying and foaming properties, and high fat-holding capacity (199%). Structural analysis revealed increased aggregation prone β -sheet content with similar molecular weight distributions, supporting improved functional behavior. The second study evaluated PAW as a novel green pretreatment for chickpea protein extraction, applied alone and in combination with conventional and DES-based methods. Optimized mild plasma conditions (20 kV, 5 min) maintained protein yields without inducing major molecular weight alterations. PAW-treated protein isolates showed increased aggregation-prone β -sheet structures in all modified extraction methods (DES-PAW, MSE-PAW and ALK-PAW) accompanied with increased surface hydrophobicity and particle size distribution. Although the molecular sub-unit distribution was similar across different treatments, there were distinct pores in PAW-treated samples in the SEM micrographs. These structural changes correlated with increased water and oil holding capacity for the modified extraction methods along with increased foaming and emulsifying properties. Overall, this work demonstrates that DES and PAW are effective, scalable, and sustainable technologies that can improve chickpea protein yield and functionality beyond conventional methods, supporting their application in functional foods, plant-based meat alternatives, and high protein beverages.

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Dedication

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List of abbreviations

ALK – Alkaline Extraction

CPI – Chickpea Protein Isolate

DES – Deep Eutectic Solvents

DSC – Differential Scanning Calorimetry

DSE – Divalent Salt Extraction

FTIR – Fourier Transform Infrared Spectroscopy

HVACP – High Voltage Atmospheric Cold Plasma

MSE – Monovalent Salt Extraction

PAW – Plasma Activated Water

PDCAAS – Protein Digestibility Corrected Amino Acid Score

PI – Protein Isolates

SDS PAGE – Sodium Dodecyl Sulphate Polyacrylamide Gel Electrophoresis

SEM – Scanning Electron Microscope

Chapter 1: Introduction

The current food system, despite significant advancements, remains far from optimal. This stems from the numerous vulnerabilities present that threaten our food security. Indeed, the COVID-19 pandemic exposed the fragility of the food system, particularly in terms of supply chains, labor, and economic insecurity, pushing us further away from achieving food security. A UN report from 2022 revealed the grim reality, indicating that about 828 million people grappled with hunger in 2021, which was a staggering increase of 150 million since the onset of the pandemic. Besides, the prices of basic food commodities quickly skyrocketed after the onset of the Russia-Ukraine war, further underscoring the system's inherent cracks and the urgency of targeted interventions.

Given the anticipated worldwide population of 9 billion by 2050, the FAO asserts that food production needs to increase by 60-70% to meet the increasing food demand (FAO, 2009). Admittedly, the very industry that nourishes us also contributes massively to greenhouse gas emissions, primarily through animal production, which continues to harm the environment and drive adverse climate change. This makes animal production an unsustainable protein source, highlighting the urgent need to explore sustainable protein sources to overturn this refutable trajectory that threatens our planet's delicate balance.

Pulses are among the most promising alternative protein sources, available in great abundance. They rank third in the most crucial crop category, following cereals and oilseeds, with a worldwide annual production of approximately 92-96 million tons (Hazra & Basu, 2023). Meanwhile, Canada ranks second among global pulse producers, accounting for approximately 9% of total production over the past decade (OECD-FAO, 2023). Among the primary pulse crops produced, dry beans are followed by chickpeas, peas, and lentils (Joshi & Rao, 2017).

One key reason for their widespread cultivation is their nitrogen-fixing ability, which improves soil fertility and increases crop yields. Moreover, pulses play substantial roles in mitigating greenhouse gas emissions and ultimately reducing global warming. Furthermore, they provide significant quantities of both macro- and micronutrients, including high-quality proteins, carbohydrates, and phytochemicals, making them household staples in most developing countries. Therefore, pulses are sustainable protein sources that could replace animal-based sources and reduce our dependency on them.

Chickpea, in particular, is one of the most popular and versatile pulses worldwide, serving as a highly nutritious crop and a sustainable source of protein. It has a high protein content (16-22%), excellent nutritional profile, and adaptability to a wide range of climates, making it a reliable crop for various regions (Summo et al., 2019). Correspondingly, chickpeas are among the most widely grown pulses worldwide, with about 11.5 million tons produced, making them widely available and a promising alternative protein source for the food industry (Merga & Haji, 2019). This has also fueled their incorporation into plant-based food products, including meat and dairy alternatives, gluten-free formulations, and high-protein snacks, catering to the evolving preferences of health-conscious and environmentally aware consumers. This growing demand underscores the need for efficient and sustainable protein extraction technologies to enhance chickpea's utility in food systems.

Conventional protein extraction methods, such as alkaline and salt-based extractions, have several limitations. These include high water use, elevated pH conditions that lead to protein denaturation, and substantial wastewater generation, all of which compromise environmental sustainability and product functionality (Hadinoto et al., 2024). Therefore, the development of green and efficient extraction methods is critical for industrial-scale applications of chickpea proteins.

Deep Eutectic Solvents (DES) represent a novel and environmentally friendly class of solvents first introduced by Abbott et al. (2003). DES consists of a mixture of hydrogen bond donors and hydrogen bond acceptors, creating a eutectic mixture with a melting point lower than the individual constituents. These organic compounds could range from choline chloride and glycerol to organic acids (e.g., citric or malic acid). DES are biodegradable, renewable, non-toxic, 100% atom economical, making them ideal candidates for sustainable extraction processes. Several studies have reported their successful use in extracting proteins from plant sources such as fava beans (Hewage et al., 2024), sesame meal (Cao et al., 2023), sea buckthorn (Lin et al., 2022), and canola meal (Karimi et al., 2024), achieving both higher protein yields and enhanced functional properties compared to traditional methods. For instance, Cao et al. (2024) demonstrated that protein isolates extracted from sesame meal using DES had a purity of 93%, surpassing the 77.1% obtained via alkaline extraction. Despite these advancements, DES has not yet been applied to chickpea protein extraction, presenting an untapped opportunity for innovation in pulse protein processing.

High-Voltage Atmospheric Cold Plasma (HVACP) is another emerging technology used to enhance the structural and functional properties of protein isolates. However, direct plasma application to food products is often associated with undesirable effects, such as discoloration and off flavors, due to uneven surface treatment (Bayati et al., 2024). This limitation has led to the development of Plasma Activated Water (PAW), a plasma-treated solvent that retains the beneficial reactivity of cold plasma while offering more uniform and sustainable treatment. PAW has demonstrated promising results in protein modification studies and in improving protein solubility in several studies, which is an essential factor in the protein extraction process (Rahman et al., 2022; Kucerovala et al., 2019; Asefi et al., 2025). One of the key challenges in plant protein processing is the inherently lower extraction yield compared to animal proteins, attributed to the complex structure of plant matrices, antinutritional factors, and limitations of existing extraction techniques. Therefore, applying PAW as a pretreatment could improve the extraction efficiency and functional quality of chickpea protein isolates.

This study aims to investigate the use of Deep Eutectic Solvents (DES) and Plasma Activated Water (PAW) to produce high-quality chickpea protein isolates with enhanced extraction yields and improved functional properties. Findings from this research are expected to contribute significantly to sustainable protein processing, offering new insights into green extraction technologies for pulse proteins.

1.1. Hypothesis

DES and PAW-modified chickpea protein isolates will exhibit improved extraction yield and superior techno-functional properties than chickpea proteins produced via conventional extraction methods (Alkaline and salt-based extraction).

1.2. Objectives

Study 1. Comparative Study on Chickpea Protein Extraction: Enhancing Techno-functionality and Quality through Deep Eutectic Solvents

- To optimize DES conditions (Water Content (%) and Liquid: Solid Ratio (w/w)) for maximum extraction yield and purity
- To determine the Protein extraction yield from DES compared to conventional methods.
- To characterize the structural and functional properties of protein isolates

Study 2: Plasma-Activated Water: A green and innovative pretreatment for improved chickpea protein quality

- To optimize plasma conditions (applied voltage and treatment time) for improved chickpea protein yield
- To determine changes in physicochemical properties of water with varying plasma conditions and their impact on the protein yield
- To investigate the impact of PAW inclusion in conventional extraction methods on the yield, structural, and functional properties of protein isolates

Chapter 2: Literature Review

2.1. Chickpea

Chickpea (*Cicer arietinum* L.), commonly known as garbanzo bean, is an important pulse crop belonging to the Fabaceae family, which also includes fava bean (*Vicia fava*), soybean, and alfalfa (Sandhu & Chaturvedi, 2025). As a widely cultivated legume, chickpea plays a significant role in global security due to its adaptability to diverse climatic conditions, high nutritional value, and ability to enhance soil fertility through nitrogen fixation. Chickpea has a rich historical background, dating back to at least 7500 BC in the Middle East, where it spread westward to Europe and eastward to Myanmar (Nair, 2019). Today, it is primarily grown in Asia, Europe, North America, and East Africa, where its resilience to drought and its ability to thrive in marginal lands make it a valuable crop for sustainable agriculture. Its cultivation is especially beneficial in semi-arid regions, as chickpea can grow with minimal water input while improving soil health (Kumar et al., 2023).

It is a self-pollinating, cool-season crop that grows best at temperatures between 15-25°C, with optimal seed germination occurring at 28-33°C (Martinez et al., 2021). The plant matures in 3-7 months, depending on environmental conditions, with flowers ranging in color from white to pink or purple. The pods that develop after flowering contain one or two seeds, the plant's primary edible part (Trancoso et al., 2021). Its root system is also well developed, featuring a central tap root with numerous nodules inhabited by *Rhizobium* bacteria, which facilitate atmospheric nitrogen fixation. This characteristic not only enhances soil fertility but also benefits subsequent cereal crops in crop rotation systems (Kebede, 2021). Additionally, chickpea's ability to tolerate cold temperatures allows it to serve as a key winter legume, filling an important gap in seasonal crop production (Maphosa et al., 2020).

Chickpea cultivars exhibit significant genetic diversity, particularly in seed size, shape, and color. The two main types of chickpeas are Kabuli and Desi. Kabuli chickpeas are large, beige-colored seeds with thin seed coats, whereas Desi chickpeas are smaller, angular, and have thick, dark brown seed coats. These physical distinctions are accompanied by differences in nutritional composition, which are influenced by factors such as genetic variation, environmental conditions, and agricultural practices (Asati et al., 2023).

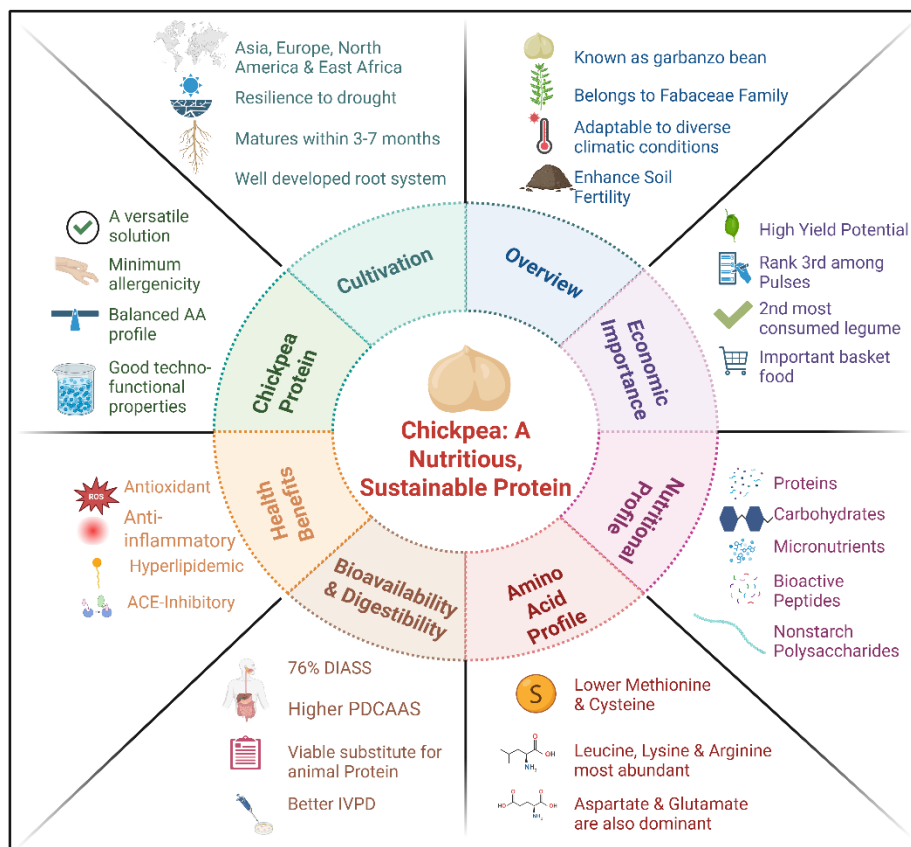


Fig 1. Comprehensive overview of chickpea as a nutritious and sustainable protein source

2.1.1. Economic importance of chickpea

Since 1961, Chickpea production has grown at an annual rate of over 1%. This increase is attributed to developments in cultivation practices, improved varieties, and increased awareness of chickpea's nutritional value (Merga & Haji, 2019). Notably, Asia is the primary contributor, accounting for about 83% of global output. Chickpeas thrive in diverse environmental conditions across 57 countries, making them a vital crop for contributing to food security (Kumar et al., 2024). Its high-yielding potential, coupled with its affordability, makes it an ideal alternative to animal protein sources.

In terms of production volume, chickpeas rank third among pulse crops, with an annual yield of about 11.67 million tons worldwide (Merga & Haji, 2019). Meanwhile, Canada is a prominent player in chickpea production, contributing around 90,000-120,000 metric tons annually to secure a spot among the top 10 global producers (USDA, 2023). However, most of this production is

devoted to exports, with minimal domestic processing. Consequently, to achieve the ambitious goal of generating nearly 4.5 billion dollars from the pulse industry, innovation must become paramount (National Research Council Canada, 2022). In this regard, key areas of innovation include developing food ingredients from protein-rich pulses, such as chickpeas, and optimizing food product formulation and processing.

Chickpeas are the second-most-consumed legume worldwide, with an average per capita consumption of 21 grams. Regions like South Asia, Latin America, Sub-Saharan Africa, and the Caribbean exhibit the highest chickpea consumption (Rehm et al., 2023). Chickpeas are among the most important basket foods in developing countries. As global food markets shift toward plant-based protein sources, chickpeas emerge as excellent candidates owing to their high protein content, making them ideal for a range of technological applications.

2.1.2. Nutritional profile of chickpea

Chickpeas have one of the highest nutritional compositions among dry edible grain legumes. As a rich source of protein, carbohydrates, and micronutrients, chickpea plays a pivotal role in addressing global food security and nutritional deficiencies. Notably, chickpea protein content ranges from 16% to 22% across genotypes (Summo et al., 2019). The amino acid profile of chickpea is high in essential amino acids, such as lysine, which is often limiting in many plant-based diets (Patil et al., 2024). However, they lack essential sulfur-containing amino acids, such as cysteine and methionine (Grasso et al., 2022). Chickpea proteins are a good source of bioactive peptides that have been shown to enhance glucose metabolism by activating AMP-activated protein kinase (Oishi et al., 2024). These peptides also exhibit anti-diabetic and anti-cancer properties, further highlighting the nutritional and therapeutic potential of chickpea proteins (Oishi et al., 2024).

The popular pulse is a rich source of micronutrients, such as Iron and Zinc, which are critical for combating micronutrient deficiencies, particularly in low-income countries (Jha et al., 2024). The Iron content in chickpea ranges from 31.2 to 81 ppm, while zinc content ranges from 32.1 to 86.1 ppm, depending on the genotype. Furthermore, chickpea contains significant amounts of bioactive compounds, such as polyphenols, flavonoids, and antioxidants, which contribute to its health-promoting properties (Jayalakshmi & Kumar, 2024).

Table 1.0 presents an overview of the proximate composition of chickpeas. Carbohydrates constitute a significant portion of chickpea seed dry weight, accounting for up to 70%. Among these carbohydrates, starch stands out. Chickpeas exhibit a C-type crystallinity pattern with large, spherical granules (Yniestra et al., 2019). Moreover, research conducted by Miao, Zhang & Jiang (2009) revealed differences between Desi and Kabuli varieties, showing that Desi had higher amylose content and higher breakdown and setback viscosities than Kabuli. Chickpeas also contain high levels of non-starch polysaccharides. It shines as a rich source of dietary fiber. Components such as hemicellulose, cellulose, and lignin contribute to their overall fiber content (Luzardo-Ocampo et al., 2020). Admittedly, adding chickpeas to diet can improve heart health, reduce the risk of diabetes, and help prevent gastrointestinal diseases.

Table 1. Proximate Composition of Chickpea (Summo et al., 2019)

Proximate Composition	Percentage (%)
Carbohydrate	45-65%
Protein	16-22%
Crude Fat	3-5%
Crude Fiber	5-13%
Ash Content	2-3%

Chickpeas serve as an indispensable source of protein, which could be instrumental in reducing dependency on animal proteins and in supplying adequate-quality protein for the growing population.

2.2. Overview of chickpea protein

Until recently, chickpea flour was the most widely used fraction of chickpeas as a food ingredient for industrial applications (Boukid, 2021). However, recent developments in the food sector and the demand for sustainable and health-conscious ingredients have shifted the spotlight to chickpea proteins. Chickpea proteins are functional, sustainable, clean-label, and healthy ingredients that are integrated into food product formulations. The incorporation of protein isolates or concentrates enables the production of functional food products with high protein content and improves their organoleptic properties.

As the food industry seeks alternatives to animal-based proteins, chickpea proteins emerge as a versatile solution. Their well-balanced amino acid profile, high bioavailability, and minimal allergenicity position make them very favorable. Chickpea proteins are available as isolates (>90% protein content), concentrates (50-90% protein content), and enriched flours (<50% protein content) (Grasso et al., 2022). Though protein isolates have the best techno-functional properties among other products, they are considered a rare commercial commodity due to the lack of sustainable protein-processing technologies (Yaputri et al., 2023). Consequently, although these protein sources are more sustainable than animal-based proteins, their use is limited by the poor structural and functional properties of chickpea proteins (Patil et al., 2024). Therefore, despite these isolates having the potential to replace soy proteins on the market, various studies show that chickpea protein has inferior properties in terms of gelation, emulsification, and solubility, which are all due to the harsh protein processing methods currently used (Yaputri et al., 2023).

2.2.1. Protein content and nutritional profile of chickpea proteins

2.2.1.1. Amino Acid Profile

Chickpea proteins have a well-balanced amino acid profile, meeting FAO/WHO requirements, though sulfur-containing amino acids are the first limiting amino acids. A study by Rodriguez-Martin (2024) found that protein concentrate had an amino acid profile that met the requirements by WHO/FAO/UNU (2007) except for the methionine and cysteine content (9.2mg/g – 14.8mg/g protein), which was lower than the required levels (20mg/g protein). Other studies also found that the only limiting amino acid was tryptophan (Goertzen et al., 2021). Therefore, complementing with other protein sources, such as cereals, is required to meet the recommended intake of essential amino acids. However, chickpeas are reported to contain higher levels of methionine and lysine than mung beans and black gram (Kaur et al., 2022). Notably, leucine (8.7%), arginine (8.3%), and lysine (7.2%) are the most abundant amino acids in chickpea proteins, as reported by Boukid (2021). Other reports indicate that glutamic acid and aspartic acid were the dominant amino acids (Onder et al., 2022). Studies have found lysine levels in chickpeas ranging from 6.5 to 7.5 g/100g of protein, enhancing its nutraceutical benefits (Zhang et al., 2022).

Several factors, including genetic factors, agronomic practices, and processing conditions, impact the amino acid profile of chickpea proteins. A study by Xio et al. (2022) to assess different Kabuli

and Desi chickpea cultivars in Xinjiang, China, found that the amino acid composition varied from 0.23 to 4.35g/100g in Kabuli chickpeas (Muying-1, Keying-1) and Desi chickpeas (Desi-1 and Desi-2). This shows the different amino acid compositions in different cultivars. Again, Verma et al. (2024) assert that chickpea genotypes grown under irrigation tend to have higher protein content than those grown in rainfed areas. Similar findings indicate that inoculation with nitrogen-fixing bacteria increased grain protein content by up to 22% (Olivera et al., 2017).

Furthermore, processing of the chickpea proteins, including Succinylation, which involves changing the protein charge distribution by converting lysine and other basic amino acids into negatively charged carboxamides, was found to improve the functional properties of the chickpea proteins; however, the amino acid profile significantly reduced from 36.65g/100g to 12.83g/100g. A combined effect with ultrasonication also resulted in a reduction of the essential and non-essential amino acids by 20.53% to 88.51% (Patil et al., 2024). This shows the significant impact of processing on the amino acid content of these proteins. Further studies on the impact of various processing conditions on the nutritional quality of protein ingredients across several industrial processes should be conducted. Again, enzymatic hydrolysis of chickpea protein isolate resulted in lower levels of cysteine and methionine contents (Goertzen et al., 2021).

Additionally, this composition significantly influences the functional and structural properties of proteins, affecting hydrophilic-hydrophobic balance, surface hydrophobicity, and electrostatic interactions (Zhou & Pang, 2018).

2.2.1.2. Bioavailability and digestibility

Chickpea proteins are also recognized for their high nutritional content, particularly their abundance of essential amino acids like lysine and a digestible indispensable amino acid score (DIAAS) of 76%, making them a viable substitute for animal proteins (Patil et al., 2024). Notably, chickpea protein isolates and concentrates exhibit higher PDCAAS values (0.73) than pea proteins (0.63) and common beans (0.68) (Espinosa-Ramirez & Serna-Saldivar, 2019). Goertzen et al. (2021) also found that the in vitro protein digestibility (IVPD) of chickpea flour was 73.8%, while the protein isolate was 82%. This confirms the suitability of chickpea proteins as nutritional ingredients for industrial applications.

Factors that influence protein digestibility include hydrophobicity, crosslinking, protein secondary structure, and antinutritional factors. Antinutritional factors, such as phytic acid and trypsin inhibitors, reduce protein digestibility by forming protein complexes and inhibiting protease activity in the gastric system (Gu et al., 2022). Studies have shown that several processing methods can improve the digestibility of proteins. Khattab & Arntfield (2009) found that soaked peas, cowpeas, and kidney beans significantly improved the IVPD; however, this led to decreased protein content owing to leaching of water-soluble proteins. Other processing methods, including cooking (Piecyk et al., 2023), extrusion (Sa et al., 2020), Microwave treatment (Sun et al., 2020), and Ultrasound (Pan et al., 2020), improved protein digestibility.

2.2.1.3. Health benefits of chickpea proteins

Chickpea proteins harbor bioactive compounds with antioxidant, anti-inflammatory, and hypolipidemic activities. Lectin, a fraction of chickpea proteins, has been shown to inhibit angiotensin-converting enzyme, which could modulate blood pressure regulation (Gupta et al., 2022). Correspondingly, a study by Martinez & Mejia (2021) to assess the impact of chickpea protein hydrolysates on type 2 diabetes showed that the hydrolysate achieved high inhibition of dipeptidyl peptidase IV, which is responsible for the inactivation of gut incretin hormones and a major contributor to type 2 diabetes development. Correspondingly, recent studies have identified 21 antimicrobial peptide candidates in chickpea protein, with Leg 1 and Leg 2 exhibiting strong bacteriostatic and bactericidal activity (Heymich et al., 2021). Therefore, Chickpea protein hydrolysates hold immense promise for innovative food applications and positively impacting health outcomes.

2.2.2. Chickpea protein processing

Chickpeas are a promising source of plant-based protein due to their balanced amino acid profile and competitive functionality. However, antinutritional factors and variable functional properties necessitate further processing to enhance protein quality and utility.

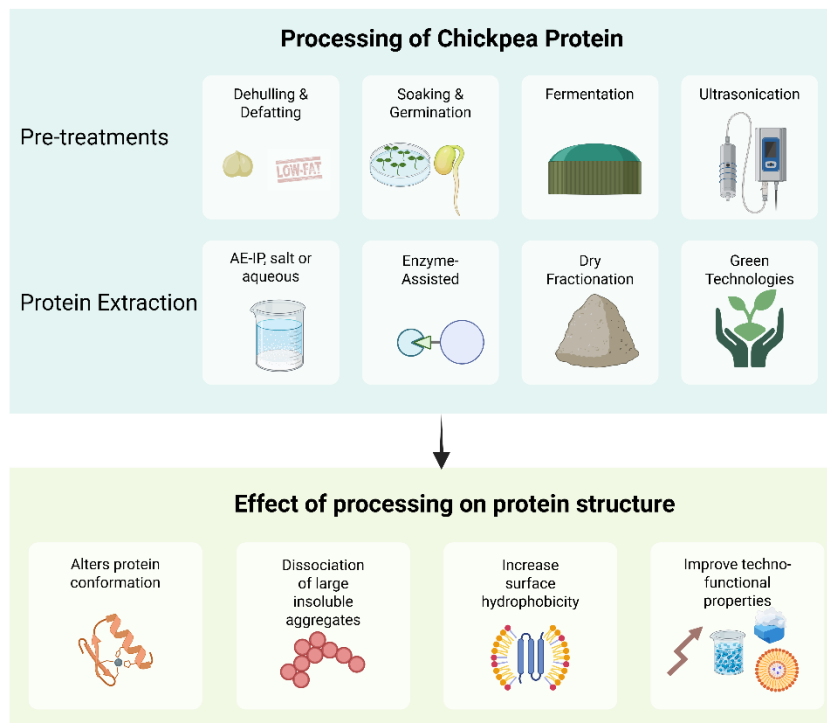


Fig 2. Processing strategies and their structural impact on chickpea proteins

2.2.2.1. Pretreatment

Pretreatments improve protein quality and extraction efficiency, enhance protein digestibility, and reduce antinutritional compounds. Common methods include dehulling, defatting, soaking, germination, fermentation, and ultrasonication. These can be broadly categorized as dry and wet pretreatment methods. The presence of polyphenols, lipids, and polysaccharides in pulse flour can interfere with protein extraction, leading to lower protein yields and quality. Thus, pretreatment methods are pivotal for enhancing protein extraction. For instance, desi chickpeas have higher polyphenol content than Kabuli types, making dehulling critical. Ultrasonication has shown promise in enhancing solubility and functionality by modifying protein structure.

2.2.2.2. Protein extraction

Chickpea proteins are typically extracted using alkaline extraction-isoelectric precipitation (AE-IP), salt extraction, aqueous extraction, or enzyme-assisted methods. These factors influence protein structure and functionality, depending on pH, temperature, and ionic strength (Aguilar-Acosta et al., 2020). Wet fractionation, such as AE-IP, yields protein isolates (>80% protein

content) but may cause extensive protein denaturation. Recent studies have shown that combining AE-IP with ultrafiltration or diafiltration increases protein yield and improves functionality (Chang et al., 2022). Other findings suggest that salt extraction using salting-in and salting-out methods yielded higher protein content and extraction yield (87.73%, 17.51%) than alkaline-extracted protein isolates (86.02%, 16.04%), as well as better functionality in terms of solubility, foaming capacity, and emulsion activity. However, AE-IP had higher protein digestibility (Higa, House & Nickerson, 2024). However, several limitations exist in the currently used technologies, especially in the production of large amounts of wastewater. Then again, AE-IP has been linked to impaired functionality of protein isolates due to the high pH required for extraction, as well as to co-extraction of polyphenols, leading to darker coloration and limiting their applications, especially in the beverage industry. Furthermore, toxic compounds such as lysinoalanine have been detected in alkaline-extracted protein isolates, underscoring the need for novel, efficient technologies to produce high-quality protein isolates sustainably (Hewage et al., 2022).

Dry fractionation is a solvent-free, energy-efficient method that uses particle size, density, and electrostatic charges for separation. It yields protein concentrates, usually the finer fraction with lower protein content compared to isolates from wet fractionation. However, not every protein ingredient used in applications has a very high protein content. For instance, meat analogues use concentrates that provide a good amount of protein, as well as starch, for better gelatinization and improved overall functionality. However, the truth remains that these ingredients have limited uses in other applications due to their lower protein content and limited functionality in emulsification and oil-holding capacity. Again, some studies have found that this method leads to concentrates with higher levels of antinutrients and off-flavors, which impact their applications, thus requiring novel and sustainable methods to overcome these barriers (Fernando, 2021).

Emerging green technologies such as reverse micelle extraction, enzyme-assisted extraction, microwave-assisted extraction, and ultrasonic-assisted extraction offer improved efficiency and functionality. Deep eutectic solvents (DES) are a standout alternative, offering non-toxic, biodegradable properties and the ability to dissolve a wide range of compounds (Hewage et al., 2022). For instance, DES-extracted fava bean proteins have shown over 92% purity with significant yield and recovery (Hewage et al., 2024). However, it has not yet been used in chickpea protein extraction. With optimized parameters (e.g., HBD: HBA ratio, water content, temperature),

DES could revolutionize chickpea protein processing. Again, Atmospheric cold plasma (ACP) is another eco-friendly, nonthermal technique originally used for sterilization that is now being explored for protein modification studies. Studies have shown that ACP improves emulsifying properties and boosts extraction efficiency when combined with enzymatic processes (Wang et al., 2024). Plasma-treated chickpea protein isolates increased protein digestibility by 11.93% while significantly improving functional properties after 30 seconds of treatment.

Table 2. Summary of chickpea processing technologies and their impact on the extraction yield and functionality

Type of Protein Processing	Chickpea Processing Technology	Impact on Extraction Yield and Functionality	Reference
Pretreatment	Enzymatic Pretreatment	- Both arabinofuranosidase and cellulase and xylanase cocktail improved recovery of protein by more than 30% with former enzyme yielding extraction efficiency about 93% - Protein extracted via enzymatic pretreatment showed improved emulsification and stability by 22% and 31% respectively while increasing the solubility and oil holding capacity by 14% and 80% respectively.	Perovic, Pajin & Antov (2022)
	Pulsed Electric Field (PEF) and Ultrasound Treatment	- Chickpea protein isolate (CPI) yield of $13.52 \pm 0.13\%$ using pulsed electric field using PEF-US, a 47.28% improvement over AE-IEP - Enhanced solubility, emulsification and foaming properties	Lai et al., (2024)

pH shifting and Cold Plasma Treatment	Treatment improved the functional properties including solubility (65.51 ± 1.07 – $73.01 \pm 0.65\%$), foaming capacity ($58.33 \pm 2.36\%$ – 2.36%) and improved in-vitro digestibility ($47.49 \pm 0.44\%$ – $55.78 \pm 0.8\%$)	Wang et al., 2024
Ultrasonic treatment	Significantly improved functional properties including emulsification and foaming stability with minimal impact on solubility and foaming capacity	Kang et al., (2022)

2.3. Structural Characterization of Chickpea Proteins

The structural characterization of chickpea proteins, from primary to quaternary structures, provides valuable insights into their functionality and potential applications in several industrial processes. Understanding these structures also provides essential information to guide modification techniques to enhance their solubility, emulsification, and digestibility.

2.3.1. Primary to Quaternary Structure

Similar to other proteins, chickpea proteins have a hierarchical structure, from the primary structure, which consists of covalent bonding of amino acids in a chain, to the quaternary structure.

2.3.1.1. Primary structure of chickpea proteins

The primary structure of proteins refers to the sequence of amino acids linked by peptide bonds. Chickpea proteins contain various amino acids, with aspartic acid and glutamic acid being the most abundant (Di Francesco et al., 2024). The amino acids form the building blocks of proteins, and the order and type of amino acids in a protein's sequence determine how it will fold into a specific 3D structure, which in turn impacts the protein's functional properties such as solubility, emulsification, and gelation. A study by Onder et al. (2022) correlated the different amino acid compositions of chickpea protein isolates with their functional properties. The emulsion capacity and foaming properties of the protein isolates were found to be negatively correlated with the

amount of acidic amino acids ($r = -0.540$) and basic amino acids ($r = -0.743$). The emulsifying stability index was positively correlated with the amount of acidic amino acids ($r=0.673$).

2.3.1.2. Secondary structure of chickpea proteins

The secondary structure of proteins refers to the arrangement of the polypeptide chains into α -helices, β -sheets, beta turns, omega loops, random coils etc. Fourier transform infrared spectroscopy and circular dichroism spectroscopy are commonly used to analyze the secondary structure of chickpea proteins. Several studies have confirmed that β -sheets are the dominant secondary structure in chickpea proteins, followed by random coils and alpha-helices (Sudarsanan, Addanki & Shanmugam, 2023). The presence of β -sheets contributes to the protein's stability and resistance to denaturation. Thus, modification techniques are used to alter the secondary structure, thereby enhancing digestibility and functional properties. For instance, Fermentation of chickpea proteins increased the α -helix content from 38.89% to 65.38% while decreasing the β -content from 40.46% to 29.76% after 24 hours (Liu et al., 2023). Nguyen et al. (2015) assert that the β -sheet is a hydrophobic structure in these proteins, often associated with aggregation and resistance to gastrointestinal hydrolysis. Conversely, Cold plasma treatment of chickpea protein isolates increased β -sheet content with increasing treatment time, from 22.07% in the control to 29.27% after 50 seconds. Meanwhile, the random and alpha helix also decreased significantly from 28.63% to 24.07% and from 37.67% to 34.77%, respectively (Wang et al., 2024). The authors explained that plasma treatment led to a loosely ordered, partially disordered structure with increasing β -sheet content. In vitro protein digestibility also showed increasing digestibility values with increasing plasma treatment time up to 40 seconds. Similarly, Ultrasonic application also led to a significant reduction in the random and alpha-helix content of chickpea proteins. It increased the β -sheet and β -turn content, due to disruption of hydrogen bonds between the carbonyl and amino groups on the polypeptide chain that stabilize the α -helix structure (Kang et al., 2022). However, if most legume seeds have relatively higher β -sheets than α -helices and are associated with low protein digestibility, how is increasing β -sheet content in the modified protein isolates leading to increased protein digestibility values?

β -sheets can be intramolecular or intermolecular depending on whether the β -strand is part of the same polypeptide chain or multiple chains. Intramolecular β -sheets, also known as aggregation-specific, are mostly found in native proteins, while Intermolecular β -sheets are found in non-native

forms (Tintor et al., 2024). A study by Blume et al. (2015), which sought to explore the relationship between protein secondary structures and increasing temperature treatment, found that increasing temperature reduced the alpha helix and intramolecular β -sheet content while increasing the intermolecular β -sheets. Similarly, Hadnadev et al. (2018) found that hemp protein isolates (HPI) produced via isoelectric precipitation had lower native intramolecular β -sheets and α -helix content than those produced via micellization (HMI), indicating extensive conformational changes in the former procedure. Accordingly, HPI had significantly better water-holding capacity, while HMI had higher fat-holding capacity. These studies show that there are conformational changes in the β -sheet structure that influence the functional and nutritional properties. Therefore, studies need to assess the different β -sheet forms to gain a better understanding of the impact of specific β -sheet forms on protein quality and to avoid any contradictions.

2.3.1.3. Tertiary structure of chickpea proteins

The tertiary structure of proteins refers to the three-dimensional conformation, stabilized by interactions such as disulfide bonds, hydrophobic interactions, and hydrogen bonds. Chickpea proteins comprise different fractions: globulins (soluble in salt), albumins (soluble in water), glutelins (soluble in acid/alkali), and prolamins (soluble in alcohol). Among these, globulins serve as the primary storage and include 11S legumin and 7S vicilin. Legumins are oligomeric proteins that exhibit molecular weights from 320-400 kDa, while Vicilins are trimeric proteins with molecular weights between 145-190 kDa (Soto-Madrid et al., 2022). Unlike legumin proteins, vicilins lack sulfur-containing amino acids, such as cysteine, and therefore lack disulfide bonds. Albumins contain essential amino acids such as threonine, lysine, and tryptophan, and they offer greater nutritional value than globulins. These constituents significantly contribute to the nutritional, techno-functional, and structural characterization of chickpea proteins. Ye et al. (2024) conducted a study to assess the structural and functional properties of chickpea albumin and globulin protein fractions. The study found that while both albumin and globulin fractions accounted for 34.97% and 34.72% of essential amino acids, respectively, albumins had a significantly higher proportion of lysine, tryptophan, and phenylalanine, while globulins had a higher cysteine content. This needs to be considered when designing extraction procedures to ensure balanced amino acid levels in protein isolates. Albumin fractions had higher solubility due to their lower molecular weight and higher proportion of hydrophilic amino acids, compared with

the higher hydrophobic content of globulins, which exhibited better emulsifying properties. These results corroborate the findings of Mundi & Aluko (2013). Therefore, the choice between globulin and albumin fraction depends on the formulation requirements and desired product attributes.

Chickpea proteins, particularly legumins and vicilins, exhibit complex tertiary structures that play a crucial role in their functionality. For instance, the 11S globulin (legumin) from chickpea seeds has been crystallized and structurally characterized. Its tertiary structure consists of an α -chain and a β -chain, with a head-to-tail association that forms threefold symmetry (Sun, Zhou & Zhang, 2022). A study by Contardo et al. (2025) found that chickpea vicilins had smaller particle sizes, a bimodal particle size distribution, and lower hydrophobicity, while having higher solubility and digestibility than the legumin fraction. The tertiary structure is discussed using techniques such as surface hydrophobicity, intrinsic fluorescence, and SDS-PAGE.

2.3.1.4. Quaternary structure of chickpea proteins

The quaternary structure refers to the assembly of multiple subunits into a functional protein. Legumin proteins, characterized by their 11S sedimentation coefficient, form a quaternary structure that is typically a hexamer composed of six subunits, each consisting of an α -chain and a β -chain linked by disulfide bonds (Rudakova et al., 2015). Meanwhile, Vicilins, characterized by their 7S sedimentation coefficient, form a quaternary structure: a trimer consisting of three subunits with a β -barrel structure (Jain, 2023). The quaternary structure is stabilized by hydrophobic interactions and hydrogen bonds between subunits (Sun et al., 2022). On the other hand, albumins are typically monomeric, although they may form dimers or higher-order oligomers. Processing techniques can influence the quaternary structure of chickpea proteins. High-pressure homogenization and atmospheric pressure plasma jet treatments have been shown to modify the quaternary structure by dissociating large protein aggregates into smaller, more soluble complexes (Wang et al., 2024; Huang et al., 2022). These structural changes can enhance the protein's properties, such as emulsification and solubility.

2.3.2. Analytical Techniques

The structural characterization of chickpea proteins is essential for understanding their functional properties, interactions, and potential applications in food and other industries. Several techniques have been developed, including FTIR, Raman, SDS-PAGE, and Surface Hydrophobicity, among

others, that describe the secondary, tertiary, and quaternary structures of chickpea proteins. These insights are essential for optimizing their functional properties for desired applications.

2.3.2.1. Fourier Transform Infrared Spectroscopy (FTIR)

FTIR spectroscopy is widely used to study protein secondary structure by analyzing the Amide I and Amide II bands. The Amide I band ($1600\text{-}1700\text{ cm}^{-1}$) is particularly sensitive to the secondary structure, as it corresponds to C=O stretching vibrations, which are influenced by the hydrogen bonding patterns in alpha helix and β -sheets. To identify specific peaks, a second-derivative analysis is performed, followed by normalization and deconvolution using Gaussian peak fitting (Sadat & Joye, 2022). The derived peaks are associated with different secondary structures reported in the literature (as shown in Table 3.0). Based on this information, the relative content of the various secondary structures can be ascertained to observe the impact of different processing methods and others on the secondary structure of the extracted proteins. The relative content of the secondary structures significantly influences the Techno functionality and quality of the protein samples.

Though some studies report similar ranges for the various secondary structures, others show slight changes. Again, while some studies go into detail to specify the types of β -sheets to understand structural modifications from the native state better, others generalize about β -forms, leading to contradictory findings. Therefore, there is a need to standardize the different ranges of secondary structures to ensure consistency and replicability of studies in the field.

Apart from the secondary structure, FTIR spectra, mostly taken from 4000 cm^{-1} to 400 cm^{-1} , also show changes in functional groups in the protein sample. For instance, peaks between 1000 and 1400 cm^{-1} indicate the presence of fluorine compounds for C-H stretching, while peaks between 1500 and 1550 cm^{-1} indicate the presence of nitro compounds and alkanes (Gyeltshen & Kamboj, 2024). These are critical in chemical modification studies to understand changes in protein structure and the functional groups present, which could, in turn, influence protein functionality and nutritional quality.

Table 3. Secondary Structures with their Associated Peaks

Secondary Structure	Wavenumber (cm^{-1})	Reference
β -sheet	1615-1637	Zhu et al., 2022

	1682-1700	Zhu et al., 2022
Pseudo- β - sheet	1613-1618	Contardo et al., 2025
Parallel β - sheet	1626-1634	Contardo et al., 2025
Anti-parallel β - sheet	1682-1695	Contardo et al., 2025
Aggregation prone β - sheet (low frequency)	1625-1615	Tintor et al., 2024
Intramolecular β - sheet	1638-1625	Tintor et al., 2024
Aggregation prone β - sheet (high frequency)	1700-1691	Tintor et al., 2024
Random coil	1636-1645	Contardo et al., 2025
	1637-1645	Zhu et al., 2022
alpha helix	1652-1661	Contardo et al., 2025
	1646-1664	Zhu et al., 2022
β - turn	1668-1675	Contardo et al., 2025
	1664-1681	Zhu et al., 2022

2.3.2.2. Raman Spectroscopy

Raman spectroscopy, a vibrational spectroscopy technique, complements FTIR by providing additional information on protein vibrational modes. Unlike IR spectroscopy, where energy absorbed is measured, Raman spectroscopy exposes high-energy monochromatic light that interacts with molecules and causes vibrational, electronic, and translational excitations (Larkin, 2017). However, the Amide I band in Raman spectra is also useful for secondary structure analysis, as it reflects the relative proportions of alpha helices and β -sheets (Sadat & Joye, 2020). Raman spectroscopy not only quantifies secondary structure but also provides key information on the strength of protein hydrophobic interactions (Kuhar et al., 2021). In a study conducted by Liu et al. (2023), Raman spectroscopy was used not only to determine secondary structural changes in fermented chickpea proteins but also to determine their side-chain conformations. The intensity ratios at 850 cm^{-1} and 830 cm^{-1} can indicate the phenolic hydroxyl hydrogen-bonding force and the microenvironmental polarity around the tyrosine residue, which can distinguish between buried and exposed tyrosine residues. When the former wavenumber is less than the latter, the residue is buried; otherwise, it is exposed (Kuhar et al., 2021). Therefore, the study found that increasing fermentation led to increased intensity at 850 cm^{-1} , while the 830 cm^{-1} band decreased, indicating an exposed tyrosinase residue. Similarly, a study by Wang et al. (2023) to understand the effects of different concentrations of chickpea protein-stabilized Pickering emulsion on the gelling properties of hairtail fish myosin gels showed, using Raman Spectroscopy, that tyrosine residues

participated in the formation of moderate or weak hydrogen bonds. This influences functional attributes, such as gel formation, as shown in the study.

2.3.2.3. Circular Dichroism Spectroscopy

Circular Dichroism Spectroscopy (CD) is another powerful tool for analyzing the secondary structure of proteins in solution. By measuring the differential absorption of left- and right-circularly polarized light, CD spectroscopy can provide information about the relative contents of alpha-helices, beta-sheets, and random coils. Far- and Near-UV Circular Dichroism was used to assess the impact of pH on the structural changes in albumin and globulin and found that albumin displayed a higher degree of alpha-helical conformation than globulin, especially at acidic and basic pH values (Ye et al., 2024). Another study using Far-UV CD spectroscopy found that the alpha helix decreased by 9.72% and 23.61%, while the β -sheets increased by 2.65% and 6.42% at pH 9 and pH 11, respectively (Zhang et al., 2023).

2.3.2.4. SDS – PAGE

Sodium Dodecyl Sulphate-Polyacrylamide Gel Electrophoresis (SDS-PAGE) is a powerful method used to ascertain the molecular weights of protein subunits. Legumin proteins consist of α and β subunits that appear as bands at ~ 37 and 19-23 kDa, respectively. Vicilin Fractions exhibit multiple subunits that appear as bands around 49, 25, 33, 19, and 15 kDa (Di Francesco et al., 2024). Albumin proteins are associated with low-molecular-weight bands. The albumin fraction corresponds to bands around 12 kDa. A study by Ramani et al. (2021) found that chickpea protein isolates showed a polypeptide band ranging from 20-75 kDa, with major bands at 20, 35, 70, and 75 kDa. The study established that the vicilin subunit (7S) is the major component of globulin in chickpea protein isolates, as the estimated molecular weights were 70.2, 50.7, 35.0, 33.6, 18.6, and 15.5 kDa. These results agree with the findings of Raut et al. (2015). 7S globulin maintains its structure mainly through hydrophobic and electrostatic interactions instead of disulfide bonds because of its limited sulfur-containing amino acids. The SDS-PAGE gel provides information on the molecular weight of protein subunits, which can indicate potential functional properties; for instance, smaller molecular weight protein isolates tend to have better solubility profiles, among other functional properties dependent on other parameters as well. However, it provides qualitative data on protein molecular weights. Techniques such as size-exclusion chromatography can be used to quantitatively determine protein molecular weight, which can help assess the impact of

hydrolytic processes, e.g., Enzymatic hydrolysis, on proteins. Particle size distribution can also determine the particle diameter of protein samples.

2.3.2.4. Scanning Electron Microscope

The scanning electron microscope (SEM) is a powerful tool for studying the internal microstructure of biological samples, such as proteins. It entails passing high-energy electrons through the sample to excite secondary electrons, which are captured and used to create an electronic image of the sample's surface topography (Ramani et al., 2021). The advantage of SEM is that it provides higher magnification (20× to 10,000×) and greater field depth, offering valuable insights into their organization and interactions (Sivakumar et al., 2022). Wang et al. (2020) conducted a study to investigate the impact of high-intensity ultrasound on the structural properties of chickpea protein isolate and found that increasing treatment time resulted in a more porous surface in SEM images. This is attributed to the mechanical and cavitation effects of high-power ultrasound, which have caused the observed porous structure and are associated with improved solubility. High-pressure homogenization yielded the opposite result: the samples treated were transformed from an irregular, rough form to a more regular, smooth form as the pressure increased (Huang et al., 2022). One disadvantage of the SEM for protein samples is that freeze-dried samples on plates often retain the plate shape and thus are not a good indicator of the protein sample's surface morphology.

2.3.2.5. Surface Hydrophobicity

The surface hydrophobicity of a protein is one of the most important indices for evaluating its conformational change. Surface hydrophobicity of proteins refers to the distribution and exposure of hydrophobic residues on the protein's surface. It plays a central role in various biological processes such as protein folding, interaction, and aggregation (Sinha et al., 2023). Most globular proteins always fold in a way that hydrophobic groups are within the structure, while the hydrophilic groups are outside in aqueous environments. Thus, the native protein structure has minimal surface hydrophobicity, which increases as the protein denatures. Wang et al. (2020) observed this effect: increasing high-intensity ultrasound on the chickpea protein isolate led to increased surface hydrophobicity. The sulfhydryl and hydrophobic amino acids were exposed as the protein structure was altered after the ultrasonic treatment. This effect also led to increased functionality, including emulsification, foaming, and gel formation, in the chickpea protein

isolates. Ghiribi et al. (2015) agreed with this finding, explaining that surface hydrophobicity influences the ability of proteins to adsorb to the oil interface, leading to improved emulsion properties. However, the study found that increasing enzymatic hydrolysis also increased surface hydrophobicity, leading to improved emulsifying properties up to a 4% degree of hydrolysis. After which the emulsifying properties began to diminish (Ghiribi et al., 2015). Similar findings were observed in treating the chickpea protein samples with high-power sonication: increasing sonication power increased surface hydrophobicity until 150W, after which it no longer increased, attributed to extensive protein denaturation and aggregation. Therefore, studies to determine the optimal surface hydrophobicity for maximum emulsifying and gelling properties should be conducted to serve as a guide for different modifications and protein extraction technologies.

Intrinsic fluorescence can also indicate the surface hydrophobicity using ANS fluorescence methods, which reflect the distribution of hydrophobic groups in proteins (Liu et al., 2015). It reflects the structural and microenvironmental changes in proteins, similar to Raman Spectroscopy. Hydrophobic amino acids such as phenylalanine, tryptophan, and tyrosine get excited at 280nm wavelengths, while only tryptophan residues get excited at 295nm (Yang et al., 2017). Using this technique, Teng et al. (2023) found that the maximum emission wavelength of chickpea protein isolate (CPI) and pea protein isolate was 318 and 327nm, respectively, which increased to 329nm for CPI after pH shifting, indicating an increase in surface hydrophobicity. Initial studies showed that pea protein had significantly higher surface hydrophobicity compared to chickpea protein. However, after simple mixing, the surface hydrophobicity of the chickpea protein isolates increased by 37.80%, thereby improving solubility and emulsifying properties (Teng et al., 2023). This shows the importance of considering the structural properties of chickpea protein isolates when tailoring their functionality for optimized food formulations.

2.3.3. Effect of Processing on Protein Structure

Various processing methods have been explored to modify the conformation of chickpea proteins, enhancing their physicochemical and functional properties. Processing methods, including thermal, nonthermal, and enzymatic modifications, can modify protein structure and improve protein quality.

Thermal processing, such as cooking and microwave heating, significantly alters the conformation of chickpea proteins. The heat treatment denatures the proteins, leading to the dissociation of large insoluble aggregates into smaller, soluble aggregates, increasing surface hydrophobicity and improving solubility and emulsifying properties. For instance, heating followed by high-pressure homogenization was observed to reduce the α -helix content while increasing the β -sheets and β -turns, thereby enhancing the solubility of chickpea proteins (Wang et al., 2025). Similarly, Hong et al. (2024) also found that cooking conditions, such as increased temperature (96°C), led to decreased peak intensities of the albumin and globulin fractions in the chromatograms from size-exclusion chromatography, while the glutelin fractions increased, indicating changes in the conformation of the chickpea proteins.

Nonthermal processing methods, including High-Pressure Processing (HPP), Ultrasonication, and Cold Plasma, have been used in several studies to modify the structure and functionality of chickpea proteins. Studies on HPP treatment of chickpea proteins found that it decreased ordered β -sheets to random coils, thereby enhancing water and oil absorption capacities (Xu et al., 2024). Similar findings were observed in ultrasonicated chickpea samples, which also showed improved in-vitro digestibility (Kang et al., 2022). Wang et al. (2023) also found that atmospheric-pressure plasma jet treatment of chickpea protein isolates resulted in extensive structural changes, with alpha-helix structures converted to random coils and beta-sheet structures, and a significant increase in protein digestibility after 30 seconds of treatment.

Other modification techniques, such as enzymatic hydrolysis and enzyme-induced polymerization, are also used. At the same time, the former leads to increased disordered structures and reduced ordered structures, such as beta sheets (Xu et al., 2020). Transglutaminase-induced protein polymerization forms high-molecular-weight polymers with increased intermolecular beta sheets (Yaputri, Feyzi & Ismail, 2023).

Apart from protein modification techniques, pretreatment and extraction technologies significantly impact the protein structure and functionality. For instance, alkaline extraction at high pH (9-11) leads to greater protein denaturation and reduced functionality.

2.4. Structure-Function Relationship in Chickpea Proteins

Food proteins are vital ingredients in food formulations due to their techno-functional properties, including acting as emulsifiers, foaming agents, gelling agents, and performing other functional roles in food systems. However, these properties result from the proteins' structural features and environmental conditions. Therefore, it is imperative to consider the functional properties as a product of the structural and extrinsic factors.

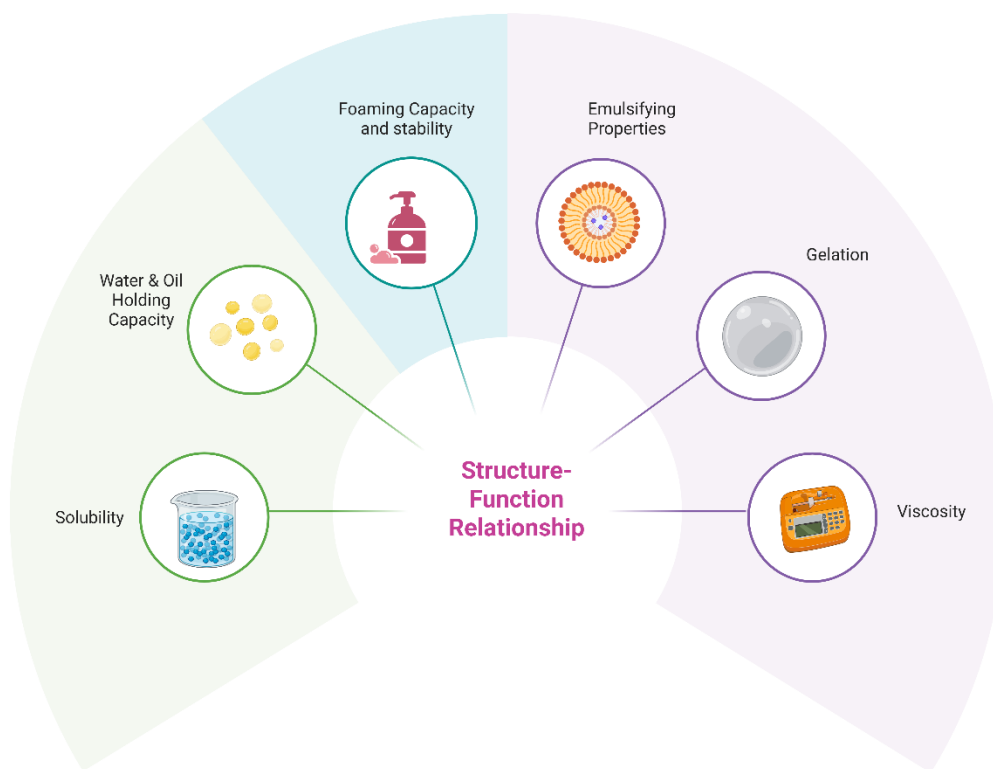


Fig 3. Structure-function relationship of chickpea proteins in food applications

2.4.1 Solubility

Solubility is a critical functional property of proteins, influencing their performance in food applications such as emulsions, foams, and gels. It depends on factors such as pH, ionic strength, and temperature. Solubility also depends on intrinsic factors such as amino acid composition, molecular size, and protein flexibility. Therefore, different protein sources display different solubility profiles. For instance, Zhang et al. (2022) found that chickpea protein concentrates had 1.5 times higher solubility than soybean protein concentrates. Contrarily, findings from Dent et al. (2023) showed that the protein solubility of soy protein and chickpea protein samples was 90%

and 80%, respectively. In order to understand the roles of the different protein subunits in solubility, Chang et al. (2022) observed that the vicilin component for both chickpea (98.19%) and pea (96.29%) had higher solubility at pH 7 than their legumin fractions, yielding solubility values of 63.51% and 68.08% respectively. This superiority is attributed to the smaller molecular weights of vicilins compared to legumins. Consequently, other studies found that a higher vicilin to legumin ratio yielded significantly higher solubility (Oluwajuyitan & Aluko, 2024). Contrary results were observed in acidic pH, where the legumin fraction (57.09-62.90%) had significantly higher solubility than the vicilin fraction (37.05%-28.39%). β -legumin is more hydrophobic than α -legumin, which is usually buried in the globulin fraction. Therefore, in acidic conditions, protein denaturation occurs, exposing the α -legumin fraction and increasing its solubility. It should be noted that the solubility of chickpea globulin was not equal to the sum of the solubility of legumin and vicilin. This can be due to the structural and conformational changes these subunits undergo at different pH values. Taherian et al. (2011) confirm this phenomenon, noting that protein solubility is strongly influenced by the balance of forces that hold the subunits together. In addition to inherent hydrogen and hydrophobic interactions, processing methods such as fermentation can improve protein solubility. For instance, Liu et al. (2023) found that the solubility of chickpea proteins increased steadily with increasing fermentation duration, reaching a maximum soluble protein content of 17.25% after 24 hours of fermentation. This can also be attributed to changes in secondary structure: the beta sheets decreased from 40.68% to 29.76%, while the alpha helix, which is an open structure, steadily increased from 38.89% to 65.38% after 24 hours of fermentation. Thus, both tertiary and secondary conformations have significant implications for protein solubility.

2.4.2. Water and Oil Holding Capacity

Water holding capacity (WHC) and Oil holding Capacity (OHC) refer to the ability of proteins to retain water and oil, respectively, against gravity. They are expressed as the amounts of water and oil, respectively, in grams bound per sample on a dry basis (Tarahi, 2024). These properties are crucial in food products, as they affect texture, juiciness, and shelf life. Several factors, including protein structure, concentration, ratios of surface hydrophobicity and hydrophilicity, plant protein source, and processing factors, influence WHC and OHC. For instance, a study by Toews & Wang (2013) found that defatted chickpea protein concentrates had higher water-holding capacity than

undefatted ones. Again, these properties are usually inversely proportional, as WHC is favored by higher hydrophilicity while OHC increases with higher surface hydrophobicity. This observation was reported in a study by Goertzen et al. (2021), in which enzymatically hydrolyzed chickpea protein isolates showed increasing water-holding capacity from 2.6 to 5.1 g/g, whereas oil-holding capacity significantly decreased from 3.6 to 2.9 g/g. Although in this study, hydrolysates had high surface hydrophobicity and a smaller molecular size, protein conformation favored improved WHC over OHC. Similar findings were reported by Perovic et al. (2022), who observed that enzymatic pretreatment of chickpea increased the WHC of protein isolates, whereas the OHC decreased compared to the control without enzymatic hydrolysis. Mesfin et al. (2021) also found that dry heating of the chickpea protein isolates led to increased WHC and decreased OHC, whereas roasting achieved the opposite, with OHC increasing while WHC decreased. The main reason for these observations is the unfolding of the protein's structure, revealing hydrophilic or hydrophobic subunits that impact the protein's functionality.

2.4.3. Foaming Capacity and Stability

Foaming Properties refer to the ability of proteins to adsorb at the air-water interface, reducing surface tension and forming robust membranes through protein-to-protein interactions. Foaming capacity refers to a protein's ability to increase foam volume by incorporating air, while foaming stability maintains foam volume over time. Notably, the albumin fraction significantly contributes to this attribute, whereas the globulin fraction does not (Yang et al., 2022). These properties are primarily predicted by protein structure, as seen in a study by Wang et al. (2022), where pH shifting and ultrasonic treatment increase the foaming capacity of chickpea protein isolates by increasing surface hydrophobicity and decreasing particle size, thereby enhancing the protein's flexibility and increasing its foaming capacity. However, the foaming stability showed an insignificant difference compared to the control group, as proteins were prone to aggregation due to increased exposure of hydrophobic groups. Similar findings were observed by Ma et al. (2023), who reported that increasing homogenization pressure up to 90 MPa significantly increased the foaming capacity, after which it decreased, whereas foaming stability decreased slightly and further decreased with increasing homogenization pressure. This was attributed to the increasing surface hydrophobicity, after which the protein was denatured and its functionality reduced. Finding a good balance between foaming capacity and stability is always preferred; thus, further studies are required to

assess the optimum structural characteristics (e.g., surface hydrophobicity and protein size and conformation) to achieve maximum foaming capacity and stability for several applications such as meringues, whipped cream, and cakes.

2.4.4. Emulsifying Properties

Protein emulsifying ability plays a crucial role in food systems. Protein isolates act as emulsifiers, helping stabilize food emulsions such as mayonnaise and milk. When emulsions are diluted in a 1% SDS solution, the protein's capacity to prevent oil droplet aggregation becomes evident. Suitably, smaller droplet sizes in protein-stabilized emulsions correlate with better emulsifying performance (Krstonosic et al., 2020). Additionally, factors such as pH, temperature, ionic strength, surface hydrophobicity, and protein solubility influence the emulsifying behavior of proteins. While the emulsifying performance of proteins measures the quantity of oil emulsified by 1g protein, the emulsifying index assesses the maximum interfacial area per gram of protein in a stable emulsion. The protein's ability to stabilize the emulsion and withstand structural changes over time is referred to as its emulsifying stability index. A study by Chang et al. (2022) found that the chickpea vicilin fraction had a significantly higher emulsifying activity index than the globulin and legumin fractions at pH 7 (55.60%) and pH 6 (66.57%). This might be due to its faster adsorption rate at the interface, attributed to its more flexible structure. Karaca, Low & Nickerson (2011) found that the emulsifying capacity of protein isolates was positively correlated with solubility and surface charge, while negatively correlated with surface hydrophobicity.

2.4.5. Gelling and Viscosity

Gelling properties play pivotal roles in various food applications, including meat analogues, jams, and bakery products. These properties arise from the denaturation of proteins, particularly globular proteins, during heating. The resulting structural rearrangements lead to uncoiled polypeptide segments that form crosslinked networks. It is influenced by the protein's surface charge, surface hydrophobicity, and free sulfhydryl groups (Tang et al., 2024). The least gelling concentration serves as a key metric for assessing this property. Notably, chickpea protein isolates have the least gelling concentration range of 14-18% surpassing their flours. Kabuli proteins yield firmer gels compared to desi flours. This property is also influenced by pH, protein concentration, temperature, and ionic strength (Boukid, 2021). Other measurable parameters include the storage modulus (G') and loss modulus (G'') to assess the viscoelasticity of the gels. A study by Wang et

al. (2023) found that adding 6-12% concentration of chickpea protein-stabilized pickering emulsion to a composite myosin gel resulted in enhanced viscoelasticity with higher G' than their corresponding G'' values. The least gelation capacity of proteins is highly dependent on pH. pH 3 produced the lowest gelling capacity (10%) compared to pH 7 and 9 (Tang et al., 2024). Interestingly, the results showed that chickpea protein isolate formed a gel at 8% at pH 7, whereas casein formed no gels at 20% protein concentration, and pea protein formed a gel at 18% protein concentration. The study also observed that none of the proteins formed a gel at pH 5, attributing this to the proteins' isoelectric points, which reduce their interactions with water and lead to random aggregation in solution. However, this aggregation should have led to gel formation, but other factors, such as temperature, protein type, and conformation, also come into play. For instance, Mengozzi et al. (2024) found that increasing temperature significantly increased the gel strength of the samples. Then again, gels formed at pH 3.0 and pH 5.0 were significantly higher than those at pH 7. Technological modifications such as Cold plasma and high-pressure processing have been used to improve the gelling properties of chickpea proteins by altering secondary structures and increasing surface hydrophobicity.

Table 4. Summary on functional properties and the underlying structural properties that influence them

Functional property	Key Structural Influences	References
Solubility	Amino Acid Composition (polar/non-polar amino acids), Surface Charge, surface hydrophobicity	Shevkani et al., (2019), Singhal et al., (2016)
Water Holding Capacity and Fat Holding Capacity	Surface hydrophobicity, pore structure, polar or hydrophobic side chains	Shevkani et al., (2019)
Emulsifying Properties	Zeta potential, surface hydrophobicity,	Shevkani et al., (2019)
Foaming Properties	Surface hydrophobicity, protein flexibility	Medrano et al., (2012)
Gelling Properties	Structural flexibility, sulfhydryl content,	Wu et al., 2019

2.5. Applications for optimized food formulations

In recent years, the protein industry has witnessed a paradigm shift driven by the rising demand for sustainable, health-conscious, and environmentally friendly food alternatives. Central to this transformation is the search for high-quality plant-based proteins that can replace animal-based proteins without compromising nutrition, functionality, or sensory attributes. Although this shift marks exciting progress in food innovation, it also brings formidable challenges. Replacing animal proteins requires alternatives that achieve the highest levels of replicability, particularly in texture, taste, and functionality. As seen in the plant-based meat industry, one of the primary reasons behind the decline in consumer acceptance of meat analogues, such as plant-based burgers, lies in their poor texture and the presence of off-flavors, which has pushed the industry to go for a high level of salts and other additives to mask the flavor. However, they are considered unacceptable to most consumers. These sensory shortcomings emphasize the urgent need to optimize formulation strategies, and chickpea proteins, given their structure-function relationships, could be utilized to offer sustainable solutions.

2.5.1. Dairy Alternatives and Meat Analogues

Chickpea proteins have emerged as valuable ingredients in the development of plant-based dairy and meat substitutes. Their emulsifying and water-binding properties make them suitable for replicating the creamy texture of dairy products. For instance, a study which sought to produce chickpea-based alternatives to cheese using both chickpea flour and chickpea protein concentrate found that the microstructural analysis showed a stronger protein matrix around the fat globules, which reduced coalescence, leading to higher levels of adhesiveness, springiness, and cohesiveness with increasing protein content (Grasso et al., 2022). To overcome issues with the calcium content of milk alternatives, Liao et al. (2024) modified chickpea protein using pH shifting to improve its solubility and calcium fortification, while ensuring good digestibility. The modification reduced the particle size from 2197.67 ± 178.2 nm to 80.2 ± 2 nm while increasing the net zeta potential from -0.48 ± 0.24 to -21.27 ± 0.65 , due to the unfolding of the secondary structure, thereby improving protein solubility and enhancing the stability of the calcium

fortification in the milk alternative. Similarly, in meat analogues, the gelling, oil- and water-holding, and emulsifying properties of these chickpea proteins play a pivotal role. Zahari et al. (2023) showed that incorporating 50% chickpea protein concentrate and 50% hempseed protein concentrate improved sensory properties and acceptability, achieving texture and taste scores of 6.4 and 6.2, respectively, suggesting it was favorably rated for its flavor. These findings were attributed to the high water-holding capacity of chickpea protein concentrate (3.38 mL/g) and its oil-holding capacity (1.82 mL/g).

2.5.2. Bakery and Snack Products

In the bakery sector, chickpea proteins offer multiple benefits, including improved texture, enhanced moisture retention, and a more nutritious profile. These properties are particularly essential in gluten-free formulations, where chickpea proteins can compensate for the absence of wheat gluten, offering high gelling, foaming, and water-holding capacities. Gupta, Liu & Sathe (2019) developed a chickpea-based snack with low fat (2.7-3.2g), high protein content (6.85-7.76g per 55g serving), and overall acceptability of 4.7 (dislike slightly) to 6.4 (like slightly). Since the study used flour, which has inherent antinutritional factors that contribute to bitterness, further studies are recommended using chickpea protein concentrates or isolates to assess their impact on snack sensory scores. For instance, incorporating chickpea protein isolates into muffins increased consumer acceptance, though they were darker, and also increased hardness (El Sohaimy et al., 2021). Improved water absorption capacity and color, achieved through novel processing conditions such as salt extraction and deep eutectic solvents, can produce high-quality chickpea protein isolates that would improve the quality of food products. Technological modifications can also be employed to improve the structural and functional properties of chickpea protein isolates.

2.5.3. Innovative and Niche Applications

Beyond conventional uses, chickpea proteins are entering innovative and emerging categories such as ready-to-drink beverages, high-protein shakes, sauces, dressings, and functional foods. However, especially in high-protein beverages and protein shakes, their solubility and stability are required to ensure their seamless incorporation. Then again, replacing whey in protein shakes requires chickpea proteins to be highly digestible, which is why whey protein is used in these products. All these requirements highlight the importance of modification techniques, such as plasma treatment, high-power sonication, and enzymatic modification, to improve the functional

properties of chickpea proteins. Given that the relationship between treatments and the protein's functional properties is only linear until a set point, after which it decreases, understanding the structural properties of chickpea that maximize the desired functional properties for the food formulation is important. Furthermore, chickpea protein's antioxidant and bioactive properties are being explored for potential health-promoting effects in functional food applications.

2.6. Challenges and Future Outlooks

Chickpea protein presents both challenges and opportunities for future research and application in the food industry. While it is recognized for its sustainability and nutritional benefits, several limitations hinder its broader adoption. Chickpea protein often exhibits poor solubility, emulsifying, and gelling properties, limiting its application in food products (Patil et al., 2024). This is due to traditional extraction methods, including alkaline extraction, which yield lower protein levels and lower protein quality than emerging techniques such as ultrasound-assisted extraction, which can be more efficient but require further optimization (Sudarsana et al., 2023). Since consumer acceptance is influenced by taste, texture, and other organoleptic qualities, addressing these factors through innovative product formulations and effective communication can enhance market penetration.

Currently, limited studies focus on the structure-function relationship of chickpeas in food product formulations due to the varied intrinsic and extrinsic factors that influence product quality. Moving forward, novel tools such as artificial intelligence and smart fingerprinting (e.g., NIR) can be tailored for protein quality determination to better understand the relationship, which could lead to optimized methods to ensure consistent, high-quality chickpea protein isolates for optimized food formulations.

To address this challenge, we can turn to green processing methods, such as deep eutectic solvents and cold plasma. By adopting these sustainable approaches, we can unlock the full potential of chickpea proteins, enhancing both their techno-functional and nutritional properties. This shift toward eco-friendly processing holds the key to a more robust and competitive plant protein industry.

Chapter 3: Comparative Study on Chickpea Protein Extraction: Enhancing Techno-functionality and Quality through Deep Eutectic Solvents

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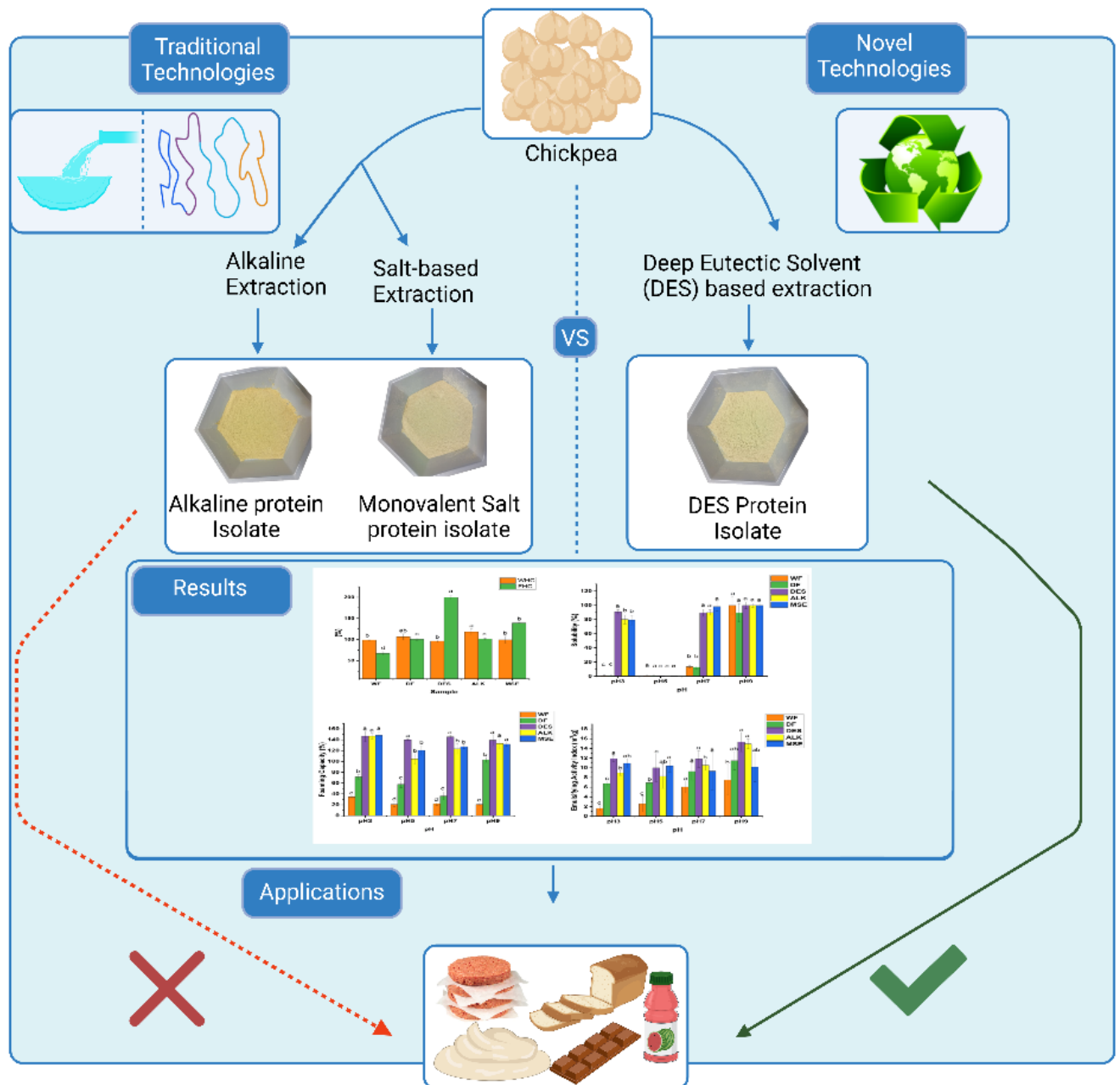
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3.1. Abstract

Traditional protein extraction methods, including alkaline and salt-based techniques, often result in significant protein denaturation, reduced functionality, and high wastewater volumes, necessitating the exploration of alternative, sustainable approaches. This study investigated the efficacy of deep eutectic solvents (DES) in extracting chickpea proteins compared to alkaline and monovalent salt extraction methods. DES solvent containing choline chloride and glycerol was optimized using a multilevel factorial design. The optimal DES extraction condition was a 15:1 Liquid: Solid ratio (w/w) and a 70% (w/w) Water substitution level. The DES-extracted protein isolates showed significantly higher protein content (87.77%), yield (53.73%), and recovery rate (12.77%) than those obtained with traditional methods. Functional characterization indicated that DES-extracted proteins exhibited enhanced solubility, emulsifying activity, foam capacity, and fat holding capacity (199%) at pH 3.0-9.0, underscoring their potential for industrial applications. Structural analysis using FTIR and SDS-PAGE highlighted distinct modifications, including increased aggregation-prone beta sheets and a similar molecular weight distribution, respectively, contributing to improved functionality. The findings suggest that DES extraction offers a sustainable and efficient method for producing high-quality chickpea protein isolates with superior functional properties, addressing the growing demand for plant-based proteins in the food industry.

Keywords: Chickpea protein, Deep Eutectic Solvent, Protein Isolate, Techno-functionality, Alkaline extraction, Salt extraction

Graphical Abstract



3.2. Introduction

Chickpea (*Cicer arietinum* L.) is a major pulse crop and a valuable source of protein rich in essential amino acids and dietary fiber (Begum et al., 2023). Often referred to as “poor man’s meat,” chickpeas provide an affordable, widely accessible protein source, particularly in developing regions. Since 1961, Chickpea production has grown at an annual rate of over 1% (Merga & Haji, 2019). This surge is attributed to advancements in cultivation practices, improved varieties, and a growing recognition of Chickpea’s nutritional benefits. Canada plays a pivotal role in global chickpea production, with a projected output of up to 327,200 tons in 2024-25, doubling 2023’s output and exceeding the five-year average (Agri-food Canada, 2024). Despite this significant production, domestic processing remains limited, with most chickpeas exported in their raw form.

There is growing emphasis on sustainability in food production, driving increased interest in plant-based proteins for their lower greenhouse gas emissions and reduced resource requirements compared to animal proteins. This shift presents an opportunity to expand pulse crop processing to meet rising global food demands. Recognizing this potential, the National Research Council of Canada highlighted key innovation areas in its 2022 report, including the development of food ingredients from protein-rich pulses and the optimization of food product formulation and processing (National Research Council, 2022). Therefore, enhancing domestic chickpea processing could add value to Canada’s pulse industry while contributing to global efforts for sustainable food production.

While Chickpea offers several nutritional and functional advantages, its high anti-nutritional content and subpar functional properties necessitate further processing. Chickpea proteins exhibit a well-balanced amino acid profile and functional properties comparable to those of commercial protein isolates (Grasso et al., 2022). As the demand for plant-based proteins grows, chickpea protein presents a viable alternative for incorporation into food products. However, the techno-functional and nutritional attributes of protein isolates can significantly be influenced by processing methods, including pretreatments, extraction approaches, and modification techniques (Ravindran, Singh & Singha, 2024).

Protein extraction from Chickpea is essential for producing protein concentrates and isolates. In both industrial and research settings, alkaline extraction and isoelectric precipitation are the most

widely used methods (Cruz Solis et al., 2023). Other protein extraction techniques include salt precipitation, aqueous extraction, and enzymatic-assisted extraction. However, these methods often lead to extensive protein denaturation, which negatively impacts the functionality (Cruz Solis et al., 2023). The functional properties of food proteins are influenced by pH, ionic strength, temperature, and other processing conditions (Aguilar-Acosta et al., 2020). Current protein extraction methods have several limitations that impact their industrial utilization. For proteins to be industrially relevant, they must exhibit high techno-functionality, including properties such as solubility and foaming capacity, depending on their intended end use and product application. These properties are influenced by factors such as the protein source, amino acid composition, and, significantly, the protein processing techniques employed (Kolpakova et al., 2018). Alkaline extraction methods subject proteins to harsh pH conditions, leading to extensive protein denaturation, a darker color, a bitter taste, and the co-extraction of polyphenols. Salt-based extraction methods also generate large volumes of wastewater, while dry fractionation methods, although energy-efficient and safer, often yield protein concentrate with low purity and yield (Hewage et al., 2022; Jain et al., 2024). Consequently, there is a pressing need to explore more sustainable protein processing technologies capable of producing highly functional protein ingredients.

Among emerging green technologies, Deep Eutectic Solvents (DES) have garnered significant attention for their sustainable and efficient properties in protein extraction. DES is a class of ionic liquid analogs composed of Lewis and Bronsted acids and bases that offers a green alternative to traditional solvents such as methanol and hexane (Smith et al., 2014; Hewage et al., 2022). DES are 100% atom-economical, eco-friendly, biodegradable, and low-toxicity (Liu et al., 2022). Previous studies have demonstrated the effectiveness of DES in extracting proteins from various sources, such as Faba beans, Oat, Rapeseed meal, and Brewer's spent grain, achieving high purity and yield. In a study by Hewage et al. (2024), faba bean protein isolates extracted using the Choline Chloride: Glycerol DES system at a molar ratio of 1:2 achieved a protein content of 92.33%, with yields and recovery rates of 65.42% and 23.15%, respectively. Other studies explored the use of the Choline Chloride and Polyethylene glycol DES system in combination with microwave treatment to extract proteins from pumpkin seeds, leading to a 93% extraction yield, while the Choline Chloride and 1,4 Butanediol system also achieved a 55.28% protein yield from Oat (Liu

et al., 2017; Yue et al., 2021). Olatunde et al., (2026) also found that choline chloride:Glycerol in a molar ratio of 1:2 obtained 72.49% protein yield from yellow pea

This presents an excellent opportunity to apply DES to chickpea protein extraction, aiming to enhance both yield and functionality. One advantage of this green solvent is that it is chemically tunable; therefore, its efficiency is influenced by factors such as the choice of DES constituents, Water content (%), temperature, and liquid-to-solid ratio (w/w) (Hewage et al., 2022). However, changing the DES compounds used has dire consequences for their toxicity and biodegradability. For instance, choline chloride: glucose and choline chloride: glycerol have been shown to exhibit low cytotoxicity, whereas choline chloride: oxalic acid exhibits moderate cytotoxicity (Ribeiro et al., 2015). Therefore, using an established chemical combination such as choline chloride and glycerol provides a safe and feasible approach for food processing, especially given that the constituents are already used as ingredients in various food applications. Since different protein sources exhibit distinct properties and behaviors, it is essential to optimize these conditions specifically for chickpea proteins.

Given that no studies have examined the application of deep eutectic solvents in chickpea protein extraction, this study seeks to explore the potential impact of DES on the processing of chickpea proteins. The study aimed to optimize DES conditions for chickpea protein extraction to maximize protein yield and content. Additionally, the study assessed the techno-functional, nutritional, and structural properties of chickpea protein isolates produced via DES compared with those produced via alkaline and salt-extraction methods.

3.3. Materials and Methods

3.3.1. Materials

Kabuli Chickpea seeds were obtained from Avena Foods in Canada. Glycerol ($\geq 99.5\%$ purity), choline chloride ($>99\%$), sodium chloride, hydrochloric acid, sodium hydroxide, and Bradford Serum Albumin Standard were sourced from Fischer Scientific (Ottawa, Ontario, Canada). Bio-Rad Laboratories (Mississauga, Ontario, Canada) supplied the precast gels (4-15%), Coomassie blue R250, Protein standards (10-250 kDa), and 2-mercaptoethanol. All the reagents purchased were of analytical grade.

3.3.2. Sample preparation

Chickpea seeds were milled using a Prater-Sterling Impact Mill with a 0.5 μm sieve. The resulting flour was defatted using a Soxhlet extractor (Electrothermal multi-position Soxhlet extraction heater, UK) for 10 h (20 cycles/hour). The defatted flour was stored at -20 °C for further analysis.

3.3.3. Experimental design – optimization of protein extraction using Deep Eutectic Solvent

A full factorial design was employed to optimize protein extraction from Chickpea using DES. Two factors, Liquid: Solid ratio w/w (X1), Water content (%) w/w (X2) were evaluated at five levels. Protein extraction was carried out using DES containing choline chloride (ChCl) and glycerol (Gly) at a 1:2 molar ratio at 50 °C for 2 h. Table 5 represents the levels of the independent variable, and Table 6 outlines the experimental design.

Table 5. Factors at five levels used in the multi-level factorial design

Factors	Levels				
	1	2	3	4	5
Liquid: Solid Ratio (w/w)	5	10	15	20	25
Water Content % (w/w)	40	50	60	70	80

Two factors and Five levels were used in the Multi-Factorial Design

Table 6. Multilevel factorial design for optimizing chickpea protein extraction using deep eutectic solvents

Runs	Liquid: solid Ratio (w/w)	Water Content % (w/w)	Protein Yield (mg/g)
1	25	40	166.12 $\pm$ 12.92
2	25	80	176.76 $\pm$ 10.75
3	25	60	183.47 $\pm$ 12.51
4	25	70	180.76 $\pm$ 2.68
5	25	50	179.24 $\pm$ 9.94
6	20	60	157.37 $\pm$ 15.93
7	20	70	141.65 $\pm$ 10.70
8	20	80	159.42 $\pm$ 1.67
9	20	50	161.12 $\pm$ 2.09
10	20	40	158.29 $\pm$ 9.95
11	15	60	116.55 $\pm$ 7.99
12	15	70	124.74 $\pm$ 8.44
13	15	50	127.03 $\pm$ 11.59
14	15	80	123.90 $\pm$ 13.87

15	15	40	104.44 ± 9.29
16	10	80	79.37 ± 10.99
17	10	70	72.43 ± 9.84
18	10	50	82.58 ± 2.85
19	10	40	85.56 ± 9.18
20	10	60	78.56 ± 7.28
21	5	50	45.66 ± 4.91
22	5	40	34.34 ± 3.03
23	5	70	45.12 ± 1.59
24	5	60	37.96 ± 2.77
25	5	80	44.43 ± 1.85

The predicted optimal protein yield from the factorial design and statistical analysis was done using MINITAB software (version 21.4.2). Protein yield was calculated in triplicate and ± indicates the standard deviation.

3.3.4. Optimization of protein extraction parameters

The protein extraction was done according to a method described by Hewage et al. (2024) with slight modifications. Defatted chickpea flour (1g) was added to the prepared DES, vortexed (10-15 s), and homogenized (Model 850, Fisher Scientific, Ottawa, Canada) at 20,000 rpm for 2 min. Then the protein extraction was performed in a shaking water bath (200 rpm) at 50 °C for 2 h, cooled to room temperature, and centrifuged (12, 000 rpm, 10 min, 4 °C). Protein content (mg/g) in the supernatant was quantified using the Bradford assay using UV-spectrophotometer (595 nm) (Genesys 180 UV-Vis Spectrophotometer, Fisher Scientific, Ottawa, ON, Canada). The yield and protein content were calculated using equations (1) and (2), respectively.

Based on the protein content (mg/g) derived, the optimum conditions were selected, also considering the efficiency of the resources involved (water content and liquid:solid ratio) while maximizing protein yield and purity. The optimum conditions were used to extract protein isolates and dialyzed for 5 days after which they were lyophilized and stored in -20 °. Protein purity, recovery rate, and protein yield are then determined using equations (1) and (2). Based on these parameters, the best condition was selected.

$$\text{Protein Yield (\%)}_{DW} = Wp \times \frac{P}{Pr} \times 100 \quad (1)$$

$$\text{Protein Recovery Rate (\%)}_{DW} = (Wp \times P)Wr \times 100 \quad (2)$$

Wp = Weight (g) of protein isolate (DW)

P = Protein content of protein isolate (DW)

P_r = Protein content of defatted chickpea flour (DW)

W_r = Weight of initial defatted chickpea flour on DW basis

3.3.5. Proximate analysis

To determine moisture content, chickpea samples were dried at 105 °C in an oven (Thermo Fisher Scientific, Pittsburgh, PA, USA) for 6 hours until they reached a stable weight. Crude protein content was assessed using the Dumas combustion method, converting total nitrogen to crude protein with a factor of 5.71. Ash content was measured by combusting the samples at 550 °C overnight in a box furnace (Model 1100, Thermo Scientific, NC, USA). Crude fat content was determined by Soxhlet extraction, as described by Nielsen (2010). All experiments were conducted in triplicate to ensure the accuracy and reliability of the results

3.3.6. Conventional protein extraction

Alkaline extraction, monovalent and divalent salt extraction were performed to compare with deep eutectic solvent extraction.

3.3.6.1. Alkaline Extraction

Alkaline extraction of chickpea proteins was done using a method described by Karaca, Low & Nickerson (2011) with slight modifications. Defatted chickpea flour was dispersed in distilled water in a ratio of 1:10 (w/v). The resulting solution was homogenized (Model 850, Fisher Scientific, Ottawa, ON, Canada) at 14,000 rpm for 1 min. The pH was then adjusted to 9.0 with 5 M NaOH and stirred at 200 rpm for 2 h at room temperature (23 °C). The alkali extract was then centrifuged at 12,000 rpm for 20 min at 4 °C. The supernatant was recovered and adjusted to pH 4.5 using 2.5 M HCl, and isoelectric precipitation was allowed overnight at 4 °C. The settled pellet was recovered and washed twice via centrifugation (12,000 rpm for 20 min). The washed pellet was redispersed in distilled water, freeze-dried, and stored at 4 °C.

3.3.6.2. Monovalent Salt Extraction

Monovalent salt extraction was performed using the method described by Karaca, Low & Nickerson (2011), with some modifications. Defatted chickpea flour was dispersed in salt solution (0.8 M NaCl) at a ratio of 1:10 (w/v). The solution was then homogenized (Model 850, Fisher

Scientific, Ottawa, ON, Canada) at 20,000 rpm for 2 min followed by stirring at 200 rpm for 1.5 h at 25 °C. Afterwards, the supernatant was recovered after centrifugation (12,000 rpm, 2 min at 4 °C), dialyzed for 5 days, freeze-dried, and stored in airtight bags for future analysis.

3.3.6.3. Divalent Salt Extraction

A 0.8 M divalent salt solution was prepared using NaCl and MgCl₂ in a 1:3 ratio. Defatted chickpea flour was dispersed in the prepared salt solution at a 1:10 (w/v) ratio. The resulting solution was homogenized (Model 850, Fisher Scientific, Ottawa, ON, Canada) at 20,000 rpm for 2 min, and extraction was done at 200 rpm for 1.5 h. Centrifugation was performed afterward to recover the supernatant, which was dialyzed for 5 days, after which it was freeze-dried and stored in airtight Ziploc bags at -20 °C. The nitrogen content of the protein extracts from conventional methods was determined using the Dumas method with a nitrogen analyzer (Rapid N exceed, Elementar, Langenselbold, Germany). Protein purity, recovery rate, and protein yield were determined by the above equations 1,2, and 3, respectively.

3.3.7. Characterization of Protein Isolates

3.3.7.1 Structural characterization of protein isolates with Fourier Transform Infrared Spectroscopy (FTIR)

The secondary structure of the chickpea protein isolates was analyzed using an Invenio FTIR spectrometer (Bruker, Billerica, MA, USA). Scans were performed across a range of 400-4000 cm⁻¹ at a resolution of 4cm⁻¹ using 120 scans. Origin 2024 software (Origin lab cooperation, MA, USA) facilitated the spectral analysis to identify the secondary structural features of the chickpea samples. Amide I peak (1600 – 1700 cm⁻¹) was analyzed using the second derivative and peak fitting to identify the secondary structures of the chickpea samples.

3.3.7.2 Morphological properties

The surface morphology of the chickpea protein isolates was examined through a scanning electron microscope (Quanta 650 FEG, FEI Company, OR USA) equipped with an Everhart-Thornley (ET) detector. Samples were pre-coated with a thin gold-palladium layer using a sputter coater and subsequently dried under nitrogen gas. The resulting images were processed and analyzed using xT microscope control software (Quanta 650 FEG, FEI Company, OR, USA).

3.3.7.3 SDS PAGE analysis

Sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE) was performed to evaluate the polypeptide profiles of the protein isolates using the Laemmli (1970) method and the method described by Hewage et al. (2024), with slight modifications. Protein solutions (4 mg/ml) were prepared in 5% SDS solution and heated at 85 °C for 5 mins. After centrifugation (20,000 rpm for 15 min at 4 °C), the supernatant was mixed with 1 mL Laemmli buffer to obtain a 2 mg/mL concentration. The reduced condition was achieved by adding 2-mercaptoethanol to the sample buffer. Protein samples and a precision standard were loaded onto a 4-15% mini-Protein TGX precast gel. Electrophoresis was carried out at 150V for 1 hour using a mini-Protean II electrophoresis cell. The gel was stained with Coomassie Brilliant Blue R-250 for 1 h and then destained using a solution containing 5% methanol and 10% acetic acid for 2 h. The resulting protein bands were visualized using the Bio-Rad ChemiDoc™ system (Bio-Rad Laboratories, CA, USA).

3.3.7.4. Particle size distribution

A static laser diffraction technique was used to measure the particle size distributions of chickpea flour and protein isolates, adapting the protocol of Alonso-Miravalles et al. (2019) with slight modifications, using the Mastersizer 3000 system (Malvern Instruments Ltd, Malvern, UK). Briefly, 1% protein suspension was prepared in distilled water at pH 7.0 and stirred for 1 h at 23 °C. The suspensions were introduced to the dispersion unit, using distilled water as the medium. The refractive and absorptive indices for chickpea proteins were set at 1.45 and 0.1, respectively, while the dispersant refractive index was 1.33. The obscuration level was adjusted to approximately 6%, and the particle size distribution was expressed using the volume-weighted mean diameter (D_{4,3}), the surface-area-weighted mean diameter (D_{3,2}), and volume percentiles (D_v 0.1, 0.5, and 0.9).

3.3.7.5. Color analysis

The color parameters (L*, a*, and b*) of chickpea samples were assessed using an LC100 Spectrocolorimeter (Lovibond, Amesbury, UK) on the Hunter scale.

3.3.8. Functional characterization of protein isolates

3.3.8.1. Solubility

Protein solubility was done using a method described by Higa, House, & Nickerson (2024) with some modifications. 0.075g of the chickpea sample was dispersed in 15 mL of distilled water. The protein solutions were allowed to hydrate overnight at 4°C, after which they were stirred for 1 hour, then the pH was adjusted to 3.0-9.0, followed by stirring at 200 rpm for 1 hour at 23 °C. The dispersions were then centrifuged, and the supernatants containing dissolved proteins were collected. Protein concentration in the supernatant was quantified using the Bradford method, with bovine serum albumin as the standard. The solubility was calculated as the ratio of the protein content in the supernatant at a specific pH to the total protein content in the sample multiplied by 100.

3.3.8.2. Water and Oil Holding capacity

The water- and oil-holding capacities of chickpea protein samples were assessed using the procedure outlined by Tontul et al. (2018), with slight modifications. A 0.1 g sample was suspended in 1.5 ml of either water or oil in pre-weighed centrifuge tubes. The tubes were allowed to stand for 30 min at 23 °C and subsequently centrifuged at 12,000 rpm for 30 minutes. The supernatant was discarded, and the pellets were weighed to determine their water- and oil-holding capacities.

3.3.8.3. Emulsification capacity

The emulsifying activity index and emulsifying stability index were evaluated based on the method described by Hou et al. (2022). A 0.5% protein solution was adjusted to pH 3.0, 5.0, 7.0, and 9.0 using 2.5 M NaOH or HCl, and homogenized at 20,000 rpm for 1 minute. A 50 µL aliquot of the resulting emulsion was mixed with 5 mL of a 0.1% sodium dodecyl sulfate solution and vortexed at 20000 rpm for 1 minute. Absorbance was measured at 500 nm using a spectrophotometer (Genesys 180 UV-Vis Spectrophotometer, Fisher Scientific, Ottawa, ON, Canada). EAI and ESI were calculated using the following equations.

$$EAI (m^2/g) = \frac{2 \times 2.303 \times A1 \times DF}{C \times \varphi \times 10000} \quad (3)$$

$$ESI (min) = \frac{A1}{A1 - A2} \quad (4)$$

A1 = Absorbance after emulsion, DF=Dilution factor, C=protein weight, φ = Volume of oil in emulsion, A2 = Absorbance of the emulsion after 10 min.

3.3.8.4. Foaming properties

Foaming capacity (FC) and foam stability (FS) were determined using a method described by Vogelsang-O' et al. (2020) with minor modifications. 1% protein solution was prepared by dispersing 0.2 g of protein isolate in 20 ml of water. The pH of the solution was adjusted to 3, 5, 7, or 9 using 2.5 M NaOH or HCl. The solutions were homogenized (Model 850, Fisher Scientific, Ottawa, ON, Canada) at 20000 rpm for 2 min. The initial foam volume (V_o) was measured after 30 seconds. The final volume (V_1) was measured after 30 min. FC and FS were calculated by using the following equations (5) and (6).

$$\text{Foaming Capacity} = \frac{V_o}{20} \times 100 \quad (5)$$

$$\text{Foaming Stability} = \frac{V_1}{V_o} \times 100 \quad (6)$$

3.3.8.5. Least gelling capacity (%)

Protein dispersions were prepared by mixing various concentrations of protein (1-20% w/v) with 1ml of 0.1M buffer solutions at different pH levels (pH 3, 5, 7 and 9). Each sample was vortexed thoroughly for 1 min and heated in a water bath at 95 °C for 1 hour. The test tubes were then rapidly cooled under running tap water and stored at 4°C overnight to allow for gel formation. Afterwards, tubes were inverted to observe gelation behavior. The Least Gelling Capacity was defined as the lowest protein concentration at which the sample did not flow upon inversion of the tube. Gels were further categorized based on physical integrity into firm gels (gel remains intact) or soft gels (gel breaks or falls apart after tapping tube). The LGC value was recorded for each pH condition accordingly.

3.3.9. Statistical analysis

The experimental data and optimization were analyzed using Minitab Software (Version 21.3.2, LLC, PA, USA). All results were analyzed at the $p < 0.05$ significance level. All analyses were performed in triplicate. Graphing was performed using Origin 2024.(OriginLab Corporation, MA, USA).

3.4 Results and Discussion

3.4.1 Optimization of protein extraction variables

Varying protein yield was observed across the different levels of the independent variables studied, as shown in Table 6. The protein yield ranged from 37.96 ± 2.77 to 183.47 ± 12.51 mg/g DW, as it was significantly influenced by the factors employed in the study. Increasing the liquid: solid ratio (w/w) from 5:1 to 25:1 resulted in higher protein yields. This is because increasing the amount of solvent ensures that all chickpea samples are fully suspended and have maximum contact with the solvent, preventing clumps and ensuring uniform extraction. Again, a higher liquid-to-solid ratio creates a larger concentration gradient between the solid and the bulk of the liquid, which provides the driving force for maximum diffusion. According to Fick's first law of diffusion, a steeper concentration gradient leads to a faster and more efficient transfer of the solute until equilibrium is attained. This explains why protein yield increased with increasing DES solvent-to-chickpea flour ratio. These results agree with those of Hewage et al. (2024), who found that increasing the liquid-to-solid ratio from 10:1 to 28:1 significantly increased protein yield from 37.47 ± 2.28 to 103.38 ± 2.31 mg/g. Similar findings were reported by Chiodza & Goosen (2024), who found higher protein yields from extraction runs with lower solid concentrations (below 30%) in the enzyme-assisted extraction system. Chouaibi et al. (2018) also observed the same trend in an alkaline extraction system, where an increased solvent ratio maximized protein yield, although this was observed only up to 30:1 v/w, after which the yield increased. This further highlights the need for optimization studies on the liquid-to-solid ratio to determine the optimal extraction condition while accounting for the system's efficiency. The regression equation below shows an empirical relationship between protein yield and the independent variables, which can predict the response within the given range.

$$\text{Protein Yield (mg/g)} = 107.91 - 64.09 X_{15} - 31.90 X_{10} + 6.08 X_{15} + 31.02 X_{120} + 58.89 X_{125}$$

The Analysis of Variance and model summary tables in Table 1S (supplementary information) provide a comprehensive evaluation of the experimental design and its ability to explain variations in the protein yield. From the ANOVA table, the model was found to be statistically significant and effectively explained variation in protein yield across the different runs in the factorial design. The independent factors showed a linear relationship with protein yield. However, increasing water

substitution level had no significant effect on protein yield, as confirmed by the Pareto chart in Fig.4 This finding could be attributed to the water substitution levels considered in this study, which ranged from 40% to 80%. Therefore, the DES prepared was less viscous than the 100% DES solution and thus had a similar level of ionicity. Studies to understand the impact of water substitution on Choline chloride and Ethylene glycol DES found that increasing the water content above 40% led to negligible changes in the ionicity especially 60% water content had ionicity of 29% which indicated that the HBA-HBD hydrogen bonding is maintained at significant dilution (Rozas et al., 2021) This also shows the possibility of achieving similar protein yields with increased water substitution level thus requiring less DES solvent in the extraction process. Further studies should be conducted to understand the impact of water-substitution level on this ChCl-glycerol DES system, including its molecular interactions and extraction capabilities.

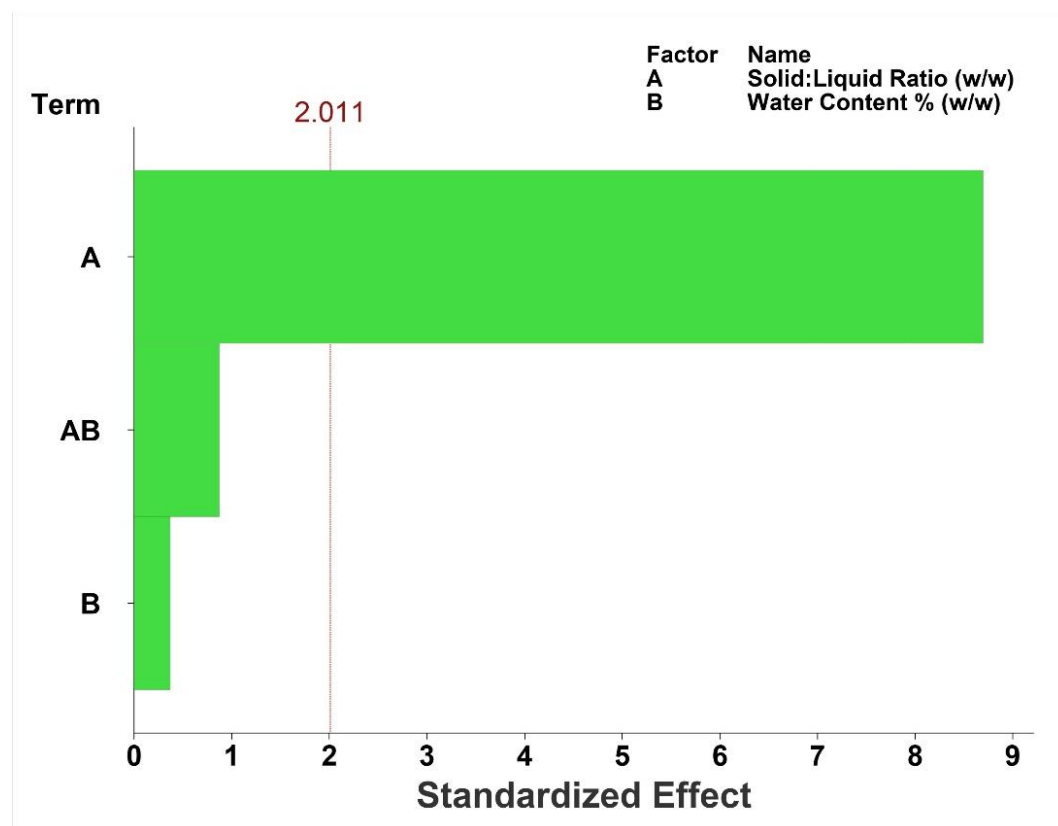


Fig 4. Pareto chart of standardized effect on protein yield (mg/g)

The R-square value suggests that the model accounts for 91.93% of the variability in the protein yield, highlighting its robustness. The adjusted R-square (91.22%) and predicted R-square (90.19%) confirm that the model not only fits the data well but also reliably predicts new observations.

Fig 5 presents the contour plots for protein yield. The results show that protein yield increases as the liquid: solid ratio rises, with the highest yields observed at ratios between 20:1 and 25:1. This observation is further corroborated by the interaction plots in the supplementary material, which reinforce the limited influence of water content on protein yield.

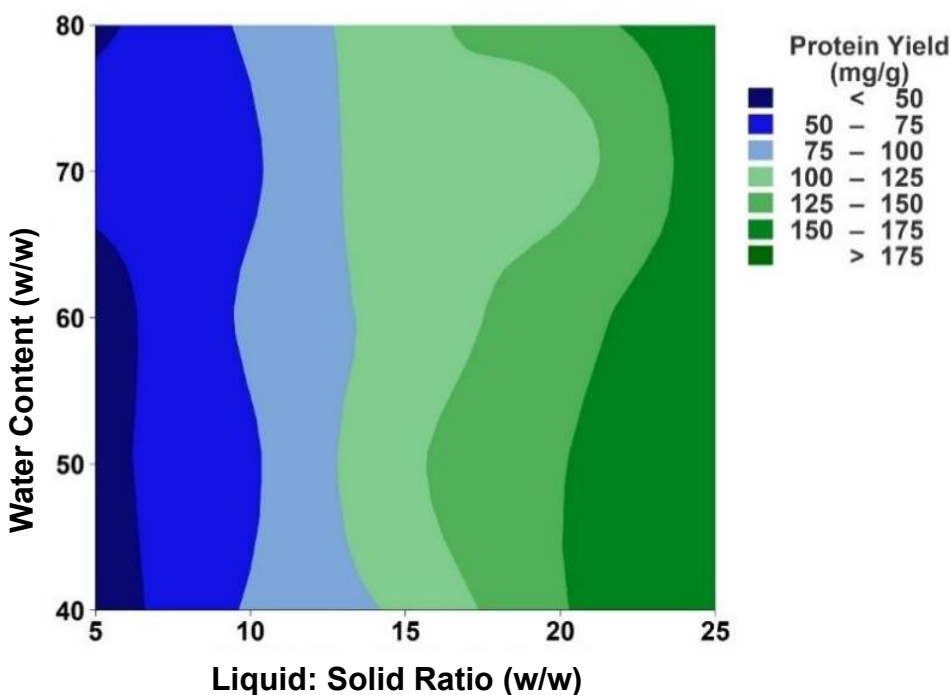


Fig 5. Contour plot of protein yield (mg/g) vs Water content % (w/w) vs Liquid: Solid Ratio (w/w).

Based on the experimental design, five conditions were selected to evaluate their effects on protein purity (%), protein yield (%), and recovery rate (%). The protein purity (%), recovery rate (%), and protein yield (%) for the various conditions are summarized in Table 7.0. Protein purity across all isolates was high, ranging from 83% to 87%. Notably, the conditions 1:15 (70%) and 1:15 (80%) exhibited significantly higher protein purity. This can be attributed to improved extraction efficiency of the protein eutectic solvent (DES) deep eutectic, facilitated by increased water

content. Recovery rates, however, were comparatively lower than those reported in other studies using DES (Ajayi et al., 2025; Bedmutha et al., 2025). This discrepancy is likely due to the lower protein content of defatted chickpea flour (22%) compared to other pulses, which typically contain higher protein levels. 1:15 (70% Water content) achieved the highest recovery rate. Lastly, protein yield ranged from 40% to 51%, with the 1:15 (70%) condition again demonstrating the highest yield. This highlights the effectiveness of this condition in maximizing protein extraction from defatted chickpea flour. Therefore, 1:15 (70%) was chosen as the optimum condition. This further assessment to select the optimum condition improved the method's efficiency by reducing the required solvent by 11% and increasing water substitution in the DES by 40% compared to previous studies (Hewage et al., 2024).

Table 7. Extraction yield (%) of chickpea proteins using different DES conditions

DES Conditions		Extraction Yield (%)		
Liquid: solid ratio (w/w)	Water Content (%)	Protein Purity (%)	Recovery Rate (%)	Protein Yield (%)
	Chickpea Flour	22.08	-	-
25:1	70	85.72±1.53 ^b	11.10±0.16 ^a	46.69±0.68 ^a
20:1	50	83.79±0.96 ^{bc}	9.66±2.12 ^a	40.66±8.93 ^a
15:1	70	87.77±0.02 ^a	12.18±0.58 ^a	51.25±2.47 ^a
15:1	80	87.78±0.73 ^a	10.89±0.20 ^a	45.83±0.82 ^a
10:1	80	85±0.22 ^b	8.58±2.83 ^a	42.04±5.95 ^a

Protein Conversion Factor : 5.71 All data were analyzed using ANOVA and Tukey test. Different superscripts within the column indicate a significant difference (P<0.05).

3.4.2. Comparative analysis of protein extraction efficiency: DES vs Conventional Methods

Table 8 summarizes the performance of different protein extraction methods, comparing their extraction yields, protein purities, recovery rates, and protein yields. Deep eutectic solvent (DES) extraction achieved the highest yield (51.25%), followed by divalent salt extraction (45.47%) while monovalent and alkaline extraction had similar lower yields (40.19% and 41.39%). The superior yield in DES extraction suggests greater protein extraction efficiency than other methods.

Similarly, DES extraction also recorded the highest protein purity (87.77%), closely followed by Monovalent Salt Extraction (87.33%). Alkaline extraction had the lowest extraction at 82.49%. High protein purity is preferred for applications such as the production of bioactive peptides, the development of high-protein snacks, and products that opt for the high protein claim.

Lastly, DES recorded a recovery rate of 12.77%, which indicates the amount of protein recovered from the entire raw material. This was significantly higher than the recovery rates attained by Monovalent and Alkaline extraction. DES extraction emerged as the most effective method across all metrics, achieving the highest yield, purity, and recovery rates. The extraction yields observed in this study are lower than yields obtained from Higa, James & Nickerson (2024), where alkaline extraction and salt extraction-dialysis resulted in 17.71 and 17.51% extraction yields from Chickpea, which could be a result of differences in the source material, pretreatments employed, and modifications in the extraction methods. The study did not indicate which nitrogen conversion factor it used for the proper assessment of purity. Protein yields obtained in this study are similar to those reported by Boye et al. (2010), who found protein yields ranging from 50.3-69.1%.

Table 8. Protein extraction yield of DES, Alkaline and Salt Extraction Methods

Extraction Method	Extraction Yield (%)		
	Protein Purity (%)	Recovery Rate (%)	Protein Yield (%)
Deep Eutectic Solvent (DES)	87.77±0.02 ^a	12.18±0.59 ^a	51.25±2.47 ^a
Monovalent Salt Extraction (MSE)	87.33±1.03 ^a	9.55 ± 0.26 ^b	40.19±1.1 ^b

Divalent Salt Extraction (DSE)	85.42±1.55 ^b	10.80±2.78 ^{ab}	45.47±11.70 ^{ab}
Alkaline Extraction (ALK)	82.49±0.60 ^c	9.84±1.32 ^b	41.39±5.57 ^b

Protein conversion factor: 5.71. All data were analyzed using ANOVA and Tukey test. Different superscripts within the column indicate a significant difference ($P < 0.05$).

3.2.1. Proximate Composition

Table 9 summarizes the compositional analysis of the different chickpea fractions, including whole flour (WF), defatted flour (DF), and protein isolates produced using different extraction methods (DES, ALK, and MSE).

The moisture content of the flours (WF and DF) was significantly higher than that of the protein isolates. This is expected, as the protein isolates undergo freeze-drying, which reduces moisture. The low moisture content of the protein isolates also has good implications for the shelf stability of the ingredients. Ash content represents the mineral content in the sample. WF and DF (2.43-2.83%) have the highest ash content, reflecting the natural mineral content of the whole and defatted flour. Other studies reported values similar to those of Dhankhar et al. (2019), who reported an ash content of around 3.30% in chickpea flour. Others also report between 3.15 and 3.48. The presence of minerals such as iron, zinc, and magnesium in chickpea flour contributes to its nutritional value and supports various health benefits (Kaur & Prasad, 2021). DES and ALK also showed ash values similar to those of the flour samples, indicating co-extraction of these minerals. Again, salts generated by these extraction methods contribute to the observed ash values. However, dialysis, which is performed for DES and MSE, significantly reduces these salts in the samples, resulting in low ash values, as seen in the results.

WF (7.73%) has the highest fat content, followed by MSE (5.29%), ALK (4.82%), DES (3.94%), and DF (3.53%). These results correspond with previous studies that found whole chickpea flour to contain 6-8% fat (Taranova et al., 2022). Pretreatments such as defatting result in higher protein yields while substantially reducing fat content.

DES (87.77%) and MSE (87.33%) had significantly higher protein content than alkaline protein isolates (82.49%) and flour samples. DF (22.05%) shows a higher protein content than WF (18.74%), as expected, since fat removal concentrates other components, such as protein. The high

protein content in isolates, especially in DES and salt extraction, demonstrates the efficiency of

Sample	Moisture	Ash	Fat	Protein	Carbohydrates
WF	8.83±0.01 ^a	2.45±0.61 ^{ab}	7.73±2.46 ^a	18.74±0.19 ^d	61.25±1.1 ^a
DF	7.77±0.18 ^a	2.83±0.01 ^a	3.54±2.46 ^b	22.05±0.05 ^c	63.81±1.81 ^a
DES	2.99±0.84 ^b	1.83±0.19 ^{ab}	3.94±0.76 ^b	87.77±0.02 ^a	3.47±1.96 ^{bc}
ALK	3.55±0.35 ^b	2.00±0.26 ^{ab}	4.82±0.79 ^{ab}	82.49±0.60 ^b	10.69±2.39 ^b
MSE	4.70±0.35 ^b	1.04±0.03 ^b	5.29±0.84 ^{ab}	87.33±1.03 ^a	1.64±2.23 ^c

the extraction method in removing non-protein components.

The carbohydrate content was highest in WF (61.25%) and DF (63.81%), both of which are unrefined. Protein isolates show significantly lower carbohydrate contents, with DES (3.47%) and MSE (1.64%) being the lowest.

Table 9. Proximate Composition of Chickpea fractions

3.4.3. Structural characterization of chickpea fractions

3.4.3.1. Secondary Structures of the Chickpea Fractions

The secondary structures of the chickpea fractions were analyzed using FTIR (Fig 6) to quantify the structural changes in the protein samples. Fig 6a shows the FTIR spectra for all the chickpea samples from 500-4000 cm⁻¹. The Amide I and Amide II bands can be observed around 1700-1600 cm⁻¹ and 1570-1510 cm⁻¹, respectively (de la Rosa-Millan *et al.*, 2018). Chickpea protein isolates exhibit more distinct peaks at Amide I and Amide II than whole and defatted chickpea flour, owing to the higher protein content in the isolates. On the other hand, the flours showed a more distinct peak in the carbohydrate region (870-1170 cm⁻¹) than the protein isolates. Amide I and Amide II regions arise due to the stretching in the C=O bond and bending vibrations of the N-H bonds, respectively. The amide I region is most important for studying changes in secondary structure, as it is susceptible to hydrogen bonding between peptide groups. The region shows overlapping component bands, including α -helix (1651 cm⁻¹), β -sheet (1623, 1634, and 1690 cm⁻¹), β -turn

(1659 and 1669 cm^{-1}), and random coils (1641 cm^{-1}) (Xu et al., 2017). Fig 2b-g presents the deconvoluted spectra for all the chickpea samples, which are summarized in Table 4. FTIR not only distinguishes between the different secondary structures, such as the α -helices, β -sheets, turns, and unordered structures, but is also able to identify different forms of the same secondary structure, such as the intramolecular and intermolecular β -sheets.

The different secondary structures and their varying relative content in the protein isolates can be observed in fig 6 and supplementary material (s3.1 and s3.2) respectively. These changes further confirm the conformational modifications induced by different extraction media and processes in the derived protein isolates. Beta sheets were significantly higher in salt-extracted protein isolates (MSE) compared to DES and alkaline-extracted protein isolates. This can be attributed to the fact that salts like NaCl can promote protein aggregation and gelation, which involves a transition from random coils and alpha-helices to more ordered, stable beta-sheet structures. Studies have shown that salt ions shield electrostatic charges between protein molecules, reducing repulsion and allowing hydrophobic interactions to drive proteins together, facilitating the formation of new hydrogen bonds and leading to more ordered, compact beta sheet structures (Zhang, 2012). These results agree with those of Ji et al. (2021), who found that increasing the NaCl concentration in soy protein isolate fibrillation from 0 to 80 mmol/L increased beta sheet content from 53.64% to 58.19%, with corresponding reductions in alpha helix and unordered coil content.

Furthermore, an in-depth analysis of the types of beta-sheet content reveals some interesting findings. As shown in Table S2, the native beta sheet structure exists as intramolecular beta sheets, whereas the aggregation-prone (low- and high-frequency) beta sheets are found in modified protein structures (Hadnadev et al., 2018). Accordingly, the flours tested, including the defatted and whole Chickpea flours, were found to contain only intramolecular beta sheets, with the other forms absent. In the protein isolates, DES-PI showed significantly higher intramolecular beta-sheet content than the salt- and alkaline-treated protein isolates, demonstrating its ability to preserve protein native structure compared to traditional extraction methods. Alkaline-extracted protein isolates also had significantly higher aggregation-prone beta sheet (low frequency), while salt-extracted protein isolates had some at higher frequencies, showing distinct differences between the protein isolates. Unordered structures were similar among the protein isolates, which were absent in the flours, showing varying levels of denaturation in the extracted isolates, which also conferred

increased functionality and improved digestibility. While the alpha helix structures were similar among DES and ALK isolates, beta turns were significantly higher in the alkaline protein isolates. This can be attributed to the high pH employed in alkaline extraction, which disrupts intramolecular hydrogen bonds while increasing repulsive forces between protein chains, leading to a more unfolded protein structure. This leads to a reduction in rigid structures like beta sheets while increasing the content of disordered structures like beta turns and random coils. These results agree with findings made by Hewage et al. (2024), where alkaline-extracted fava bean protein isolates had higher beta turns, 19.71% compared to DES, 17.11, and the Fava bean flour, 16.68%.

Another interesting finding is that, from Table S3, significant changes in the alpha helix content between the whole chickpea flour and defatted chickpea flour can be observed. The whole chickpea flour was observed to have a higher alpha helix content compared to the defatted flour, which corresponded to an increase in the unordered helix content in the defatted flour, while it was reduced in the whole flour. This confirms the conformational changes that pretreatments such as defatting confer on the protein structure. Further studies should be conducted to assess the impact of these changes on nutritional and functional properties, as well as the modifications induced by different defatting techniques.

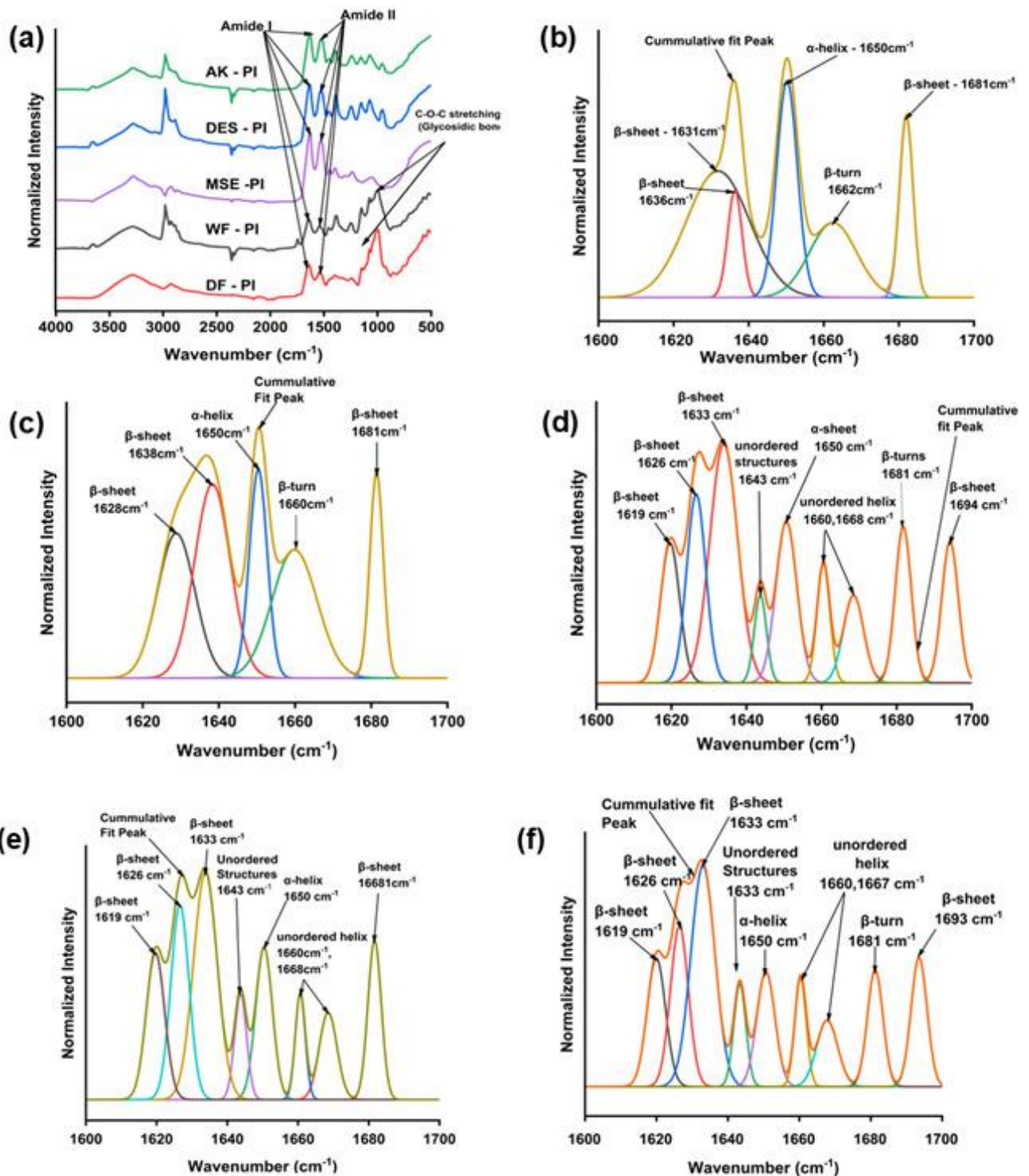


Fig 6. FTIR spectra for chickpea protein fractions (a) FTIR spectra for all chickpea fractions, (b-g) peak fitting of amide I with the different secondary structures (b) Whole Chickpea flour (c)

Defatted Chickpea flour (d) Deep eutectic Solvent extracted - PI (e) Alkaline Extracted - PI (f)
Monovalent salt extracted - PI

3.4.3.2. Molecular Weight Profile

Fig 7 shows the SDS-PAGE profiles of the chickpea protein fractions along with standard markers under reducing and non-reducing conditions. The molecular distribution of the subunits ranged from 10 to 100 kDa. Major polypeptide bands were obtained at 20, 35, 50, and 75 kDa. Most bands were attributed to the polypeptide constituents of legumin and vicilin, owing to globulins (42%) representing the major protein fractions in chickpeas (Ramani et al., 2021). The SDS patterns were similar for the samples except for two bands with molecular weights between 75 to 100 kDa. Both Monovalent and Divalent Salt extracted protein isolates did not have any band above 75 kDa, while the other protein isolates and flour samples had band intensities close to 100 kDa. These results are coherent with findings made by Ramani et al., (2021) where salt-extracted chickpea protein isolates had bands ranging within 20 – 75 kDa. This could be attributed to the salt solubilization method at low ionic strength employed by the two techniques, where smaller proteins, which have better solubility, are extracted rather than the larger protein sub-units. Three bands were attributed to globulin, including Convicilin, legumin and Vicilin at 66, 55 and 46 kDa. Under the reducing condition, legumin acidic and basic subunits were identified at 35 kDa and 25 kDa, respectively. The albumin fraction can also be seen at 14kDa for most of the samples except the salt-extracted protein isolates. This could be because of the extraction mechanism used by the salt extraction, which extracts more of the globulin portion, which is salt soluble, than the albumin portion, which is water soluble. This could influence some functional properties such as the foaming and emulsification characteristics of the protein isolates (Chukwuejim, Kadam & Aluko, 2025).

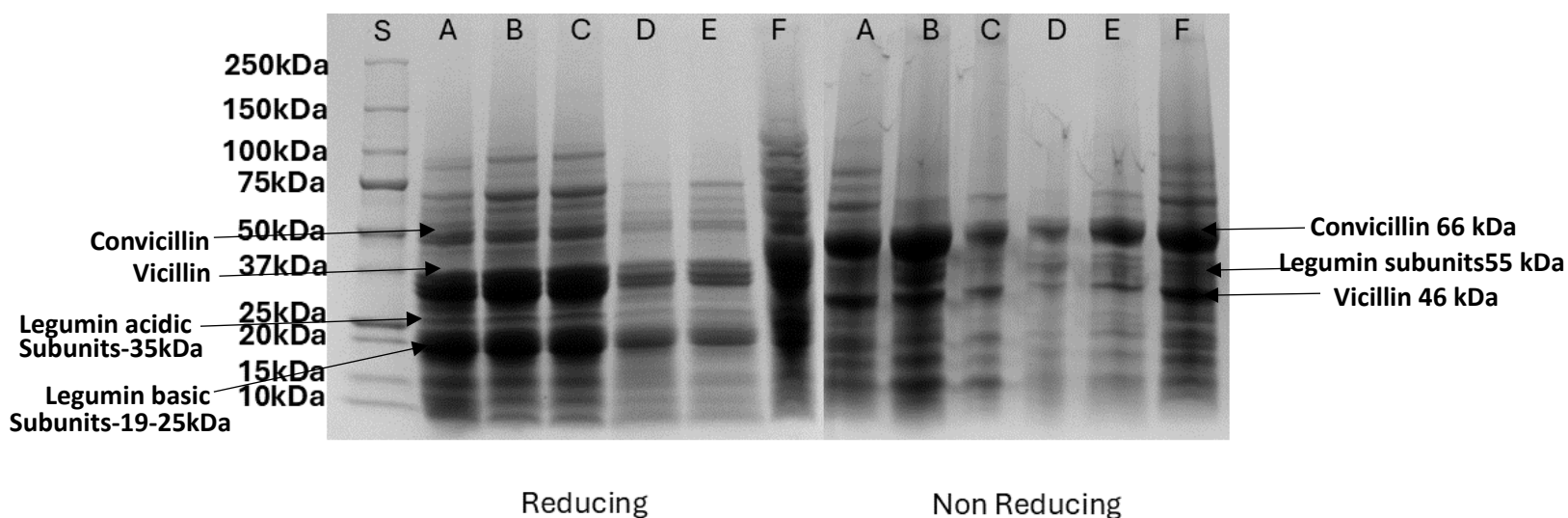


Fig 7. SDS-PAGE profile of chickpea fractions. S: Molecular weight marker. A: Defatted flour; B: DES protein isolates; C: Alk protein isolates; D: Monovalent Salt protein isolates; E: Divalent Salt protein isolates; F: Whole flour

3.4.3.3. Scanning Electron Microscope

The morphology of chickpea flour samples and protein isolates obtained via DES, Alkaline, Monovalent, and divalent salt extraction is shown in Fig 8 below. The flour samples exhibited several visible spherical microstructures, indicating the presence of starch, whereas the protein isolates did not display such large spherical structures in their SEM images. This is because chickpea flour has a high starch content (50%), while protein isolates have little starch. The protein isolates mostly show thin flakes, which are due to freeze-drying of the samples after protein extraction. The DESb showed a smoother surface at $\times 2000$ magnification, whereas the other protein isolates, such as ALKb, MSEb, and DSEb, exhibited a rougher surface under the same magnification. The images show differences in protein structure resulting from different protein processing techniques. Though the DES-protein isolates have a smoother surface, they still exhibit visible pores, as do the other protein isolates, which could significantly influence functional properties such as protein solubility and hydration.

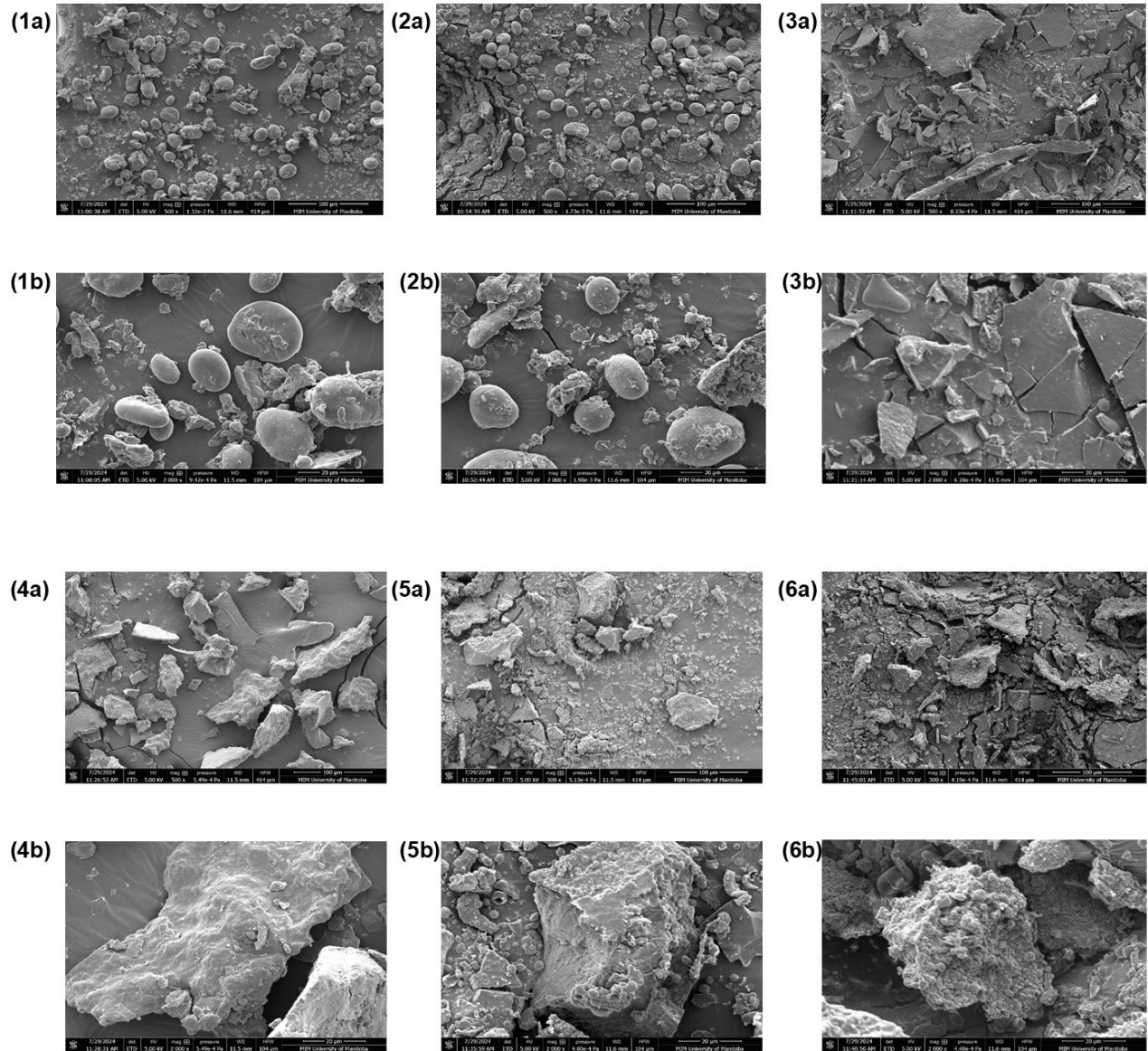


Fig 8. SEM images for chickpea fractions; (a): $\times 500$ magnification; (b) $\times 2000$ magnification. 1 = Whole chickpea flour; 2 = Defatted chickpea flour; 3 = Deep eutectic solvent protein isolates; 4 = Alkaline extracted protein isolates; 5 = Monovalent salt

3.4.3.4. Particle Size Distribution

The particle size distributions for the chickpea fractions are shown in Table 10 below. The distribution curves (S3.3) are monomodal for the protein isolates and bimodal for the flour samples. This indicates the different particle sizes present in the flour samples, while the protein

isolates showed a more uniform distribution. Uniformity is crucial for applications that require consistent texture and performance. The differences in particle size could be due to the milling process or the inherent characteristics of chickpea flour.

The D [4,3] values range from 64.4 μ m to 107 μ m, indicating a broader particle size in the alkaline protein isolates and a finer size in divalent salt extracted protein isolates. The D [3,2] values also suggest that DES has the highest value at 24.7 μ m, while divalent salt has the lowest at 11.57 μ m. This result corroborates the SDS-PAGE results, which showed lower-molecular-weight subunits in salt extracts compared to the other extraction methods. The Dx (90), (50), and (10) values further emphasize the variability within the samples. Similar findings were reported by Tanger, Mertens, and Kulozik (2022), who found that salt-extracted proteins had a smaller particle size than alkaline-extracted protein isolates.

This detailed examination of particle-size distributions highlights the intricate nature of chickpea fraction compositions, which could be pivotal for tailoring them for specific industrial applications. The larger particle sizes in some samples might make them suitable for coarse-textured products, while the smaller, more uniform sizes in others could enhance smoothness in other food formulations. The particle size distribution greatly affects several protein functionalities, including solubility and emulsifying capacity

Table 10. Particle size distribution for chickpea fractions

Particle Size distribution						
(μ m)	WF	DF	DES	ALK	MSE	DSE
D [4,3]	72.3 $\pm$ 2.89 ^a	98.2 $\pm$ 4.16 ^b	104 $\pm$ 0.58 ^c	107 $\pm$ 1.15 ^c	75.3 $\pm$ 2.22 ^a	64.4 $\pm$ 2.18 ^d
D [3,2]	13.5 $\pm$ 0.15 ^a	17.9 $\pm$ 0.15 ^b	24.7 $\pm$ 0.26 ^c	16.8 $\pm$ 0.1 ^d	13.3 $\pm$ 0.1 ^a	11.57 $\pm$ 0.15 ^e
Dx (90)	206 $\pm$ 10.26 ^a	316 $\pm$ 16.70 ^b	219 $\pm$ 2.08 ^a	242 $\pm$ 4.16 ^c	172 $\pm$ 4.04 ^d	139 $\pm$ 3.79 ^e
Dx (50)	29.7 $\pm$ 0.21 ^a	25.18 $\pm$ 4.85 ^b	83.3 $\pm$ 0.49 ^c	81.1 $\pm$ 0.59 ^c	48.8 $\pm$ 0.23 ^d	44.5 $\pm$ 0.75 ^e
Dx (10)	6.01 $\pm$ 0.076 ^a	8.77 $\pm$ 0.092 ^b	13.5 $\pm$ 0.21 ^c	6.64 $\pm$ 0.06 ^d	5.54 $\pm$ 0.02 ^e	4.69 $\pm$ 0.09 ^f

All data were analyzed using ANOVA and Tukey test. Different superscripts within the column indicate a significant difference (P<0.05). WF=Whole Flour, DF=Defatted Flour, DES=Deep eutectic solvent -PI, ALK=Alkaline extracted protein isolates, MSE=Monovalent Salt extracted protein isolates, DSE=Divalent Salt extracted protein isolates

3.4.3.5. Colour

Table 11 presents color measures of the different chickpea fractions in the CIE Lab color space, with L^* , a^* , and b^* representing lightness, redness/greenness, and yellowness/blueness, respectively. These values provide insights into the visual characteristics of the samples, which are influenced by their composition and the extraction methods used.

Defatted flour (DF) exhibited the highest lightness value ($L^* = 85.70$), followed by whole flour (WF), which was slightly darker than defatted flour owing to the presence of additional components like oil and pigments. Among the protein isolates, DES recorded the highest L^* value, while the alkaline-extracted protein isolates had the lowest L^* value. This could be because these plant materials contain phenolic compounds, which, under high alkaline conditions, oxidize rapidly, generating reactive intermediates called quinones. These quinones then interact with and polymerize with the proteins, leading to the darker color observed in this study. This is further evident in the b^* and a^* values, where Alk showed strong yellow and red hues. Lightness is a quality attribute that can be incorporated into several food products, as it affects their appearance.

On the other hand, Alk exhibited the highest b^* and a^* values, showing a strong yellow and red hue. This could result from the alkaline conditions altering protein structure, co-extracting other compounds, and enhancing yellow and redness tones while reducing the lightness value. A similar trend was observed in studies by Karimi et al. (2024), where not only did natural deep eutectic solvent produce a lighter canola protein isolate, but increasing the pH to 12 from 9 also significantly reduced lightness from 71.36 to 62.50, with increased redness as well. .

Table 11. Colour measurements for chickpea fractions

Sample	L^*	a^*	b^*
DES	81.13±1.60 ^a	2.03±0.42 ^c	16.40±0.69 ^c
ALK	71.57±0.81 ^c	4.00±0.44 ^a	22.63±0.91 ^a
MSE	75.47±0.85 ^b	3.00±0.27 ^b	18.97±0.81 ^b

All data were analyzed using ANOVA and Tukey test. Different superscripts within the column indicate a significant difference ($P < 0.05$). DES=Deep eutectic solvent extracted protein isolate; ALK=Alkaline extracted protein isolate; MSE=Monovalent Salt extracted protein isolate

3.4.4. Functional Characterization of Chickpea Fractions

3.4.4.1 Solubility

The solubility profiles of the different chickpea fractions are presented in Fig. 9 below. Protein solubility is a key functional property that influences other characteristics, such as emulsification and foaming. It provides valuable insight into the potential of the proteins as industrial ingredients for various applications (de Almeida et al., 2024). Significant differences in the solubility of the extracted proteins and flours were observed over a pH range of 3-9. Whole and defatted chickpea flours showed lower solubility at all pH levels compared to the protein isolates. This can be attributed to the presence of polyphenols and other components, such as starch and lipid complexes, which reduce protein solubility (Ramani et al., 2021).

The solubility curves of the protein isolates followed the characteristic V-shape, as observed in several studies, with the highest solubility at pH 9: 48.70% and 77.42% for defatted flour and other fractions, respectively. The lowest solubility values, ranging from 0.42–2.75%, were recorded at pH 5, close to the isoelectric point of the protein isolates. Notably, DES-extracted protein isolates and salt-extracted protein isolates had significantly ($p < 0.05$) higher solubility at pH 3.0 compared to alkaline protein isolates, although alkaline protein isolates had significantly higher protein solubility at pH 9. This variation in solubility among the protein isolates is likely due to differences in the relative content of hydrophilic and hydrophobic amino acid residues, influenced by the extraction media and processes used (Tang *et al.*, 2021). The reduced protein solubility of alkaline at pH3 can be attributed to the extensive aggregation that occurred during solubilization step in alkaline extraction meanwhile at pH9 which is significantly above the isoelectric point confers a net negative charge on the proteins which results in a strong electrostatic repulsion which enhances water binding and keeps these denatured, flexible molecules highly soluble at alkaline often resulting in a superior solubility.

The solubility data from this study are comparable to those reported by Ramani et al. (2021), who found that chickpea flour exhibited 31.69% and 87.17% solubility at pH 9 and 11, respectively. Additionally, alkaline-extracted chickpea protein isolates were reported to have solubility values of 51.41% at pH 11. In another study by Onder *et al.* (2022), chickpea protein isolates produced via alkaline-isoelectric precipitation had solubility profiles ranging from 90.2% to 99% at pH 9, and from 14.6% to 39.4% at pH 3. The particle size distribution of the protein isolates was found

to highly influence solubility behavior, as salt-extracted isolates with the smallest particle size exhibited improved solubility at pH 3 and pH 9. These comparisons highlight the superior solubility profiles of the chickpea fractions in this study. Protein solubility plays a crucial role in achieving desirable sensory qualities in food applications, such as beverages. Therefore, protein fractions from chickpea, especially DES-protein isolates, are promising functional ingredients for industrial use.

3.4.4.2 Water Holding Capacity & Fat Holding capacity

Water and Oil Holding Capacity refers to a material's ability to retain water and fat against gravity. These functionalities significantly influence the texture and mouthfeel of food products, such as bread. The water- and oil-holding capacities of the chickpea fractions are shown in Fig 8 below. The water holding capacity for the alkaline-extracted chickpea protein isolates (ALK) was significantly ($p < 0.05$) higher than the other protein fractions, except the defatted flour (DF). This result can be attributed to extensive protein unfolding and denaturation during alkaline extraction, which exposes more hydrophilic amino acid residues and creates a loose, open protein matrix that can trap and bind large amounts of water.

Unlike the findings of Ramani et al. (2021), who reported that chickpea protein isolates had significantly higher water-holding capacity than their flours, this study found that the water-holding capacity of the protein isolates was similar to that of whole and defatted chickpea flours. Factors that influence water-holding capacity include the presence of carbohydrates, protein conformation, and amino acid composition. Moreover, a multiple regression predictive model developed by Onder et al. (2021) identified the amount of uncharged polar amino acids as the major factor (71.2%) influencing the protein hydration profiles across the different chickpea fractions.

DES-extracted protein isolates had significantly higher oil-holding capacity (FHC) than the other chickpea fractions. Surface hydrophobicity, particle size, and amino acid composition can influence FHC. Chickpea protein isolates had significantly higher FHC than their corresponding flours, except for the alkaline-extracted protein isolates. These findings align with previous studies (Withana Gamage et al., 2011; Ramani et al., 2021), which reported that protein isolates had higher FHC than the flours. Defatted flour exhibited significantly higher FHC than whole flour, demonstrating the importance of various processing techniques to enhance the functionality of

flour fractions for use as food ingredients. These results underscore the potential of these protein isolates, particularly DES protein isolates, for use in plant-based protein analogs and emulsions.

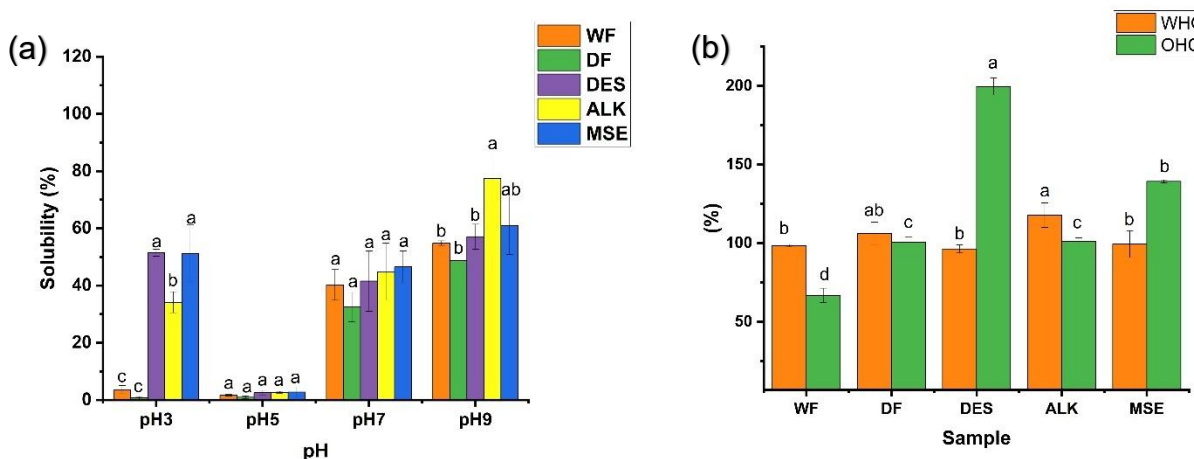


Fig 9. (a) Solubility, (b) Water Holding Capacity (WHC) and Fat Holding Capacity (FHC) profiles for chickpea fractions; (WF-Whole Chickpea flour; DF- Defatted Chickpea flour; DES- Deep Eutectic Solvent; ALK – Alkaline extracted pI; MSE - Monovalent Salt – PI. Superscripts above bars show significant differences among samples for each functionality.

3.4.4.3 Emulsifying properties

The emulsifying activity index (EAI) and emulsifying stability index (ESI) indicate the ability of the chickpea fractions to facilitate the mixing of two immiscible phases, which is crucial for the stability and organoleptic properties of various food products, such as mayonnaise, milk, and fat replacers. The EAI and ESI profiles of the chickpea fractions are presented in Fig 10 below. DES extracted protein isolates showed significantly ($p < 0.05$) higher EAI ($11.98 \pm 0.59 \text{ m}^2/\text{g}$) than the other chickpea fractions ($1.66\text{-}10.71 \text{ m}^2/\text{g}$), except for monovalent salt extracted proteins ($10.89 \pm 0.99 \text{ m}^2/\text{g}$). These results are consistent with findings on oil and water-holding capacity, where DES-extracted protein isolates had the highest FHC. EAI is influenced by the ability of proteins to adsorb oil droplets and form densely packed layers in emulsions. Proteins that have good EAI have good solubility, balanced amino acid composition, and relatively lower molecular weight. Onder et al. (2022) found that the amount of hydrophobic amino acids is the most critical parameter (93.7%) in influencing the emulsification properties of a protein sample. The whole chickpea flour

exhibited the lowest EAI, followed by defatted flour, likely due to the presence of protein complexes, such as lipoproteins and glycoproteins, which reduce protein flexibility and hinder adsorption at the oil-water interface, thereby lowering EAI.

The emulsifying index for all samples increased with increasing pH, which is consistent with findings by Ramani et al. (2021) owing to increased protein solubility. One key observation was that increasing pH reduced the EAI of monovalent salt-extracted proteins (MSE), whereas DES-extracted protein isolates maintained significantly higher EAI values across the pH range studied. It demonstrates the versatility of DES protein isolates and their potential application in various food products. EAI values in these protein isolates were lower than those reported in the literature, which could be attributed to differences in methodology and cultivar variation.

For the emulsifying stability index, an interesting observation was that whole flour, despite having the lowest EAI values, exhibited significantly higher ESI values than the other chickpea fractions, potentially due to its higher fat content, which may have contributed to the calculated ESI. Another possible explanation is the presence of lipoproteins in the whole flour, which could play a key role in stabilizing oil droplets within the continuous phase of the emulsions. A study by Zhang et al. (2024) found that high-density lipoproteins enhance emulsification capacity and stability, especially at alkaline pH levels, leading to a more uniform droplet size. However, in this study, the impact of lipoproteins on EAI was not evident. Further studies are needed to understand the mechanism underlying lipoproteins' role in emulsion stability and their potential applications in food products.

3.4.4.4 Foaming Properties

Foaming capacity and stability demonstrate the ability of proteins to form foams by adsorbing at the air-water interface, reducing surface tension, and thereby creating strong membranes through protein-protein interactions (Day, 2013). The foaming capacity and stability profiles of the chickpea fractions are shown in Fig 10 below. Foaming capacity among samples ranged from 20.78% to 146% with both whole flour (WF) and defatted flour (DF) having the lowest foaming capacity across the pH ranges tested. DES samples displayed significantly higher foaming capacity than other samples from pH 5 – pH 9, while ALK displayed the lowest foaming capacity at pH 5. This increased foaming capacity can be attributed to the higher alpha-helix content in DES-protein

isolates, coupled with improved oil-holding capacity, which allowed greater protein flexibility and increased interaction with air bubbles to form the foam structure.

Different protein isolates and chickpea flours showed distinct trends across the pH range regarding foaming properties. While Monovalent salt and Alkaline extracted protein isolates' foaming capacity decreased significantly at pH 5, DES remained consistently high across the pH ranges tested, likely due to differences in surface properties and amino acid composition. Grasso *et al.* (2022) reported that albumin fractions have superior foaming capacity and stability compared to globulins. The foaming capacities observed in this study were higher than those reported by Onder *et al.* (2022), whose chickpea protein isolates had foaming capacities ranging from 60% to 70%. DES showcased impressive foaming capacity and versatility across a wide pH range.

The flours had higher foaming capacity as they produced a low volume of foam and thus remained for a longer period. Stability was similar, except at pH 7, where DES was significantly higher than for the other protein isolates. Foam stability is influenced by surface hydrophobicity and the number of hydrophobic amino acids in the protein sample. Onder *et al.* (2022) found that the presence of acidic amino acids (79.1%) negatively correlated with foaming capacity and stability. The foaming properties of these protein isolates suggest they could be useful in food products that require foaming characteristics, such as ice cream and butter.

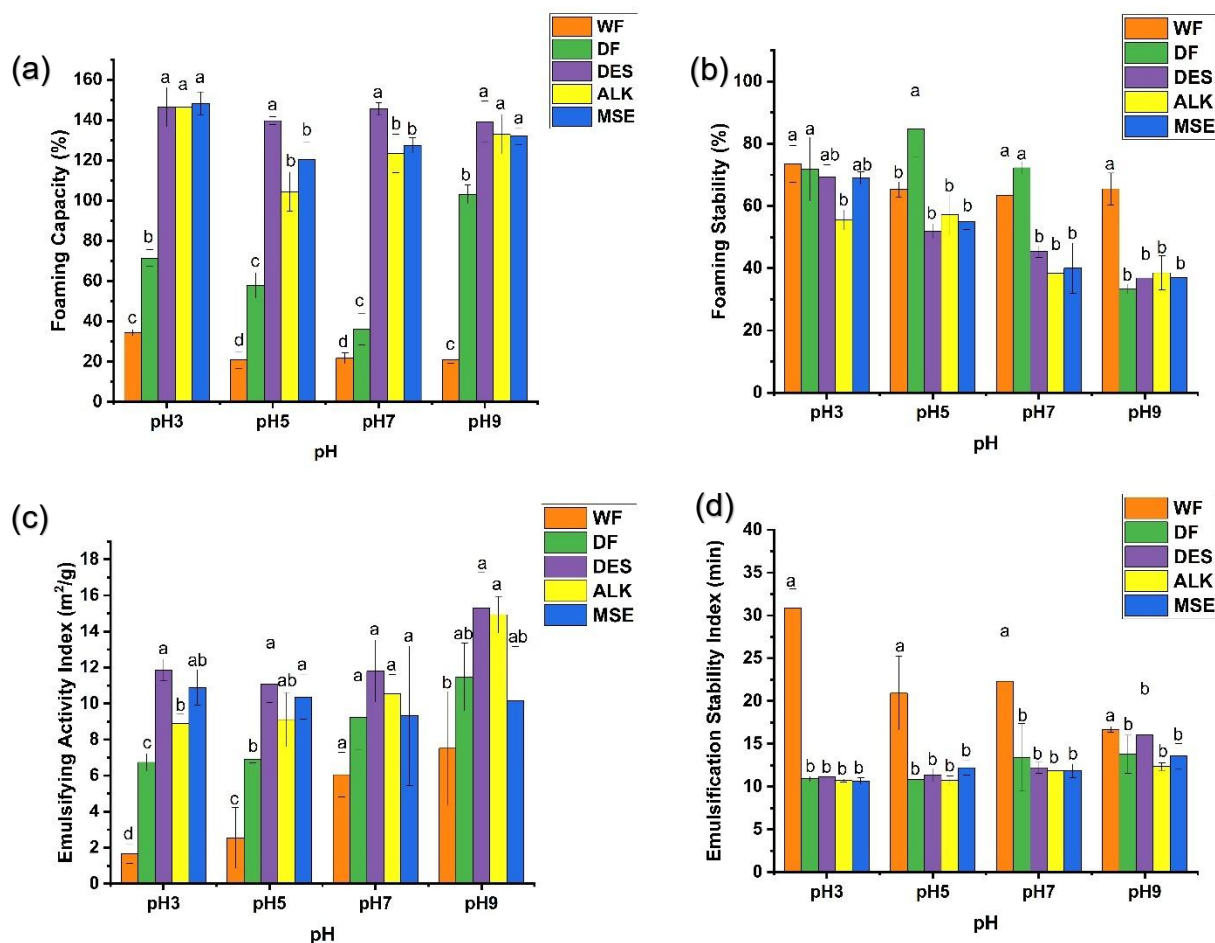


Fig 10. (a) Foaming Capacity (b) Foaming Stability (c) Emulsifying Activity Index (d) Emulsifying Stability Index Foaming and emulsifying properties for Chickpea fractions. WF – Whole flour; DF – Defatted flour; DES – Deep eutectic Solvent extracted protein isolates; ALK – Alkaline extracted Protein Isolates; MSE – Monovalent Salt extracted protein isolates

3.4.4.5. Least Gel Capacity

The least gel capacity (LGC) for the chickpea fractions is shown in Table 12.0 below. Protein isolates formed gels at lower concentrations across different pH levels, whereas chickpea flour required a 10% concentration at pH 7.0 to form a gel. This result agrees with the findings of Kaur and Singh (2007), who reported that chickpea flours had an LGC of 10%-14%. However, they found that the chickpea protein isolates had the least gelling capacity of 14-18%. In contrast, DES-extracted protein isolates had a minimum gelling concentration of 6% across pH 3-7, demonstrating impressive versatility and potential applications in food products.

At pH 5, monovalent salt-extracted protein isolates and alkaline-extracted isolates had the least gelling capacity of 4% and 5%, respectively, demonstrating an impressive ability to form gels at that low protein concentration. The extraction method plays a crucial role in determining the gelation behavior of protein isolates (Grasso et al., 2022). Gelation properties are influenced by factors such as disulfide bonds, hydrophobic amino acids, polypeptide heterogeneity, and hydrophobic interactions between protein subunits (Onder et al., 2023).

The protein secondary structure, especially the β -sheet content, also influences protein gel formation. Shevkani et al. (2015) reported that 7S globulin, which contains a higher proportion of beta sheets (38.6%), exhibited better storage modulus (G') compared to pea proteins, which contain both 7S and 11S globulins (30% beta sheets). The intermolecular hydrogen bonds between beta sheets, arranged in either parallel configuration, act as junction zones that stabilize the gel structure. Therefore, it was no surprise that salt-extracted protein isolates (MSE) had the lowest gelling capacity of 4% at pH 5, due to their significantly higher beta-sheet content.

An interesting finding is that all the protein isolates studied had their lowest gelling concentration at pH 5. This is due to protein aggregation driven by intermolecular sulfhydryl-disulfide reaction and hydrophobic interactions. At higher pH levels, that is, pH 9, proteins have a greater net charge, which keeps them soluble and prevents aggregation. This aligns with Langton *et al.* (2020), who observed that the onset of G' increase occurred at lower temperatures at pH 5 compared to pH 7, and that gel samples at pH 5 exhibited a higher G' than those at pH 7..

Table 12. Least gel concentration of chickpea fractions

Concentration (%)	Whole Chickpea Flour				DES				Alkaline ChpI				Monovalent Salt Extracted Proteins			
	pH3	pH5	pH7	pH9	pH3	pH5	pH 7	pH9	pH3	pH5	pH 7	pH9	pH3	pH5	pH 7	pH9
1	NG	NG	NG	NG	NG	NG	NG	NG	NG	NG	NG	NG	NG	NG	NG	NG
2	NG	NG	NG	NG	NG	NG	NG	NG	NG	NG	NG	NG	NG	NG	NG	NG
3	NG	NG	NG	NG	NG	NG	NG	NG	NG	NG	NG	NG	NG	NG	NG	NG
4	NG	NG	NG	NG	NG	NG	NG	NG	NG	NG	NG	NG	NG	SG	NG	NG
5	NG	NG	NG	NG	NG	NG	NG	NG	NG	SG	NG	NG	NG	SG	NG	NG
6	NG	NG	NG	NG	SG	SG	SG	NG	NG	SG	NG	NG	NG	SG	NG	NG
7	NG	NG	NG	NG	SG	SG	SG	NG	NG	SG	NG	NG	NG	SG	NG	NG
8	NG	NG	NG	NG	SG	SG	SG	NG	NG	SG	SG	SG	SG	SG	NG	NG
9	NG	NG	NG	NG	SG	SG	SG	NG	NG	SG	SG	SG	SG	SG	NG	NG
10	NG	NG	SG	NG	SG	SG	SG	NG	NG	SG	SG	SG	SG	SG	NG	NG
11	NG	NG	SG	NG	HG	SG	SG	NG	NG	SG	SG	SG	SG	SG	SG	NG

12	NG	NG	SG	NG	HG	SG	SG	SG	NG	SG	SG	SG	SG	SG	SG	SG
13	NG	NG	SG	NG	HG	SG	SG	SG	NG	SG	SG	SG	SG	SG	SG	SG
14	NG	NG	SG	NG	HG	HG	HG	SG	SG	SG	SG	HG	SG	SG	SG	SG
15	NG	SG	SG	NG	HG	HG	HG	HG	SG	SG	SG	HG	HG	SG	SG	SG
16	NG	SG	SG	NG	HG	HG	HG	HG	SG	SG	SG	HG	HG	SG	SG	SG
17	HG	SG	SG	NG	HG	HG	HG	HG	SG	SG	SG	HG	HG	SG	SG	HG
18	HG	SG	SG	NG	HG	HG	HG	HG	SG	SG	SG	HG	HG	HG	SG	HG
19	HG	SG	SG	SG	HG	HG	HG	HG	SG	SG	HG	HG	HG	HG	SG	HG
20	HG	SG	SG	SG	HG	HG	HG	HG	SG	SG	HG	HG	HG	HG	SG	HG

No gel

Soft gel

Hard gel



3.5. Conclusion

This study highlights the potential of deep eutectic solvents (DES) as a sustainable and efficient alternative to conventional methods for extracting proteins from chickpeas. DES-extraction of chickpea protein isolates exhibited significantly higher extraction yields, including protein purity, protein yield (%), and recovery rate (%). Protein isolates showed substantial changes in secondary structure, with increased aggregation-prone beta sheets and disordered structures. DES-protein isolates also showed improved functional properties, including enhanced solubility, higher oil-holding capacity, higher foaming and emulsification capacities, and consistently lower least gelling capacity across the pH levels tested. These findings demonstrate the impressive potential of DES-extracted chickpea protein isolates as excellent techno-functional ingredients for various industrial applications. Furthermore, DES's eco-friendly nature aligns with the global focus on sustainable food production. More studies should be conducted on the safety and scalability of deep eutectic-based extraction for producing high-quality chickpea protein isolates.

3.6. Contribution of Authors

Kofi Oduro: Designing the experiment, Data Analysis and writing – original draft. Anurudikka Hewage: writing – review and editing. Yu Zhao: writing – review and editing. Dr. Nandika Bandara: conceptualization, supervision, funding acquisition and writing – review and editing

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Chapter 4: Plasma Activated Water: A Green and Innovative Pretreatment for Enhancing Chickpea Protein Extraction

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4.1 Abstract

The growing demand for sustainable, high-quality dietary proteins has intensified interest in plant protein sources and environmentally friendly extraction technologies. Chickpea (*Cicer arietinum*) is a promising protein source due to its high protein content and functional versatility; however, protein yield and functionality are strongly influenced by extraction and pretreatment strategies. This study investigated the potential of plasma-activated water (PAW) as a novel, green pretreatment for chickpea protein extraction, both as a standalone extraction medium and in combination with conventional alkaline, monovalent salt, and deep eutectic solvent extraction methods. Plasma conditions were optimized using a multi-factorial design, with mild treatment parameters (20 kV, 5 minutes) identified as optimal for maximizing protein yield. The incorporation of PAW maintained protein extraction yields across all extraction methods without inducing major alterations in the molecular weight distribution, as confirmed by SDS-PAGE analysis. FTIR analysis revealed increased β -turns and aggregation-prone β -sheets, corroborated by increased particle size distributions in the PAW-modified protein isolates. Functionally, PAW-treated protein isolates exhibited increased surface hydrophobicity, improved water and oil holding capacity, foaming capacity, and stability, as well as improved emulsifying activity index. This work highlights PAW extraction as a scalable, sustainable pretreatment that enhances protein functionality while seamlessly integrating into existing extraction workflows.

Keywords: Plasma Activated Water, Cold Plasma, Protein Extraction, Protein Functionality

4.2 Introduction

The term “protein” derives from the Greek word *proteios*, meaning “of primary importance,” to highlight the fundamental role of these macromolecules in physiological function. Proteins are composed of amino acids, which provide the body with essential nitrogen, sulfur, and hydrocarbon skeletons that cannot be synthesized endogenously or replaced by other nutrients such as carbohydrates and lipids (Wu, 2016). Amino acids also serve as precursors for vital low-molecular-weight substances, such as creatine and glutathione, and are essential for the synthesis of peptides and other proteins. For instance, glutamine provides ATP to lymphocytes and macrophages, which contribute to immune responses (Li et al., 2007). These benefits, which include promoting cardiovascular health, improving cognition, and preserving muscle mass, underscore the importance of protein in the daily diet, as reflected in recommendations such as the Canada Food Guide, which suggests that high-protein foods should constitute a quarter of each meal (Health Canada, 2025).

Consequently, the demand for dietary protein is rising dramatically, with projections indicating a 48-60% increase in global demand for meat and milk between 2005 and 2050 (Alexandratos & Bruinsma, 2012; Kim et al., 2019). However, conventional animal agriculture is unsustainable for meeting this demand, especially as the global population approaches 10 billion amid significant climate challenges and resource depletion. Ruminants like cattle and buffalo currently supply 45% of the world’s animal protein, while chicken and pigs contribute 31% and 20% respectively (Mottet et al., 2017). This production consumes one-third of global cereal harvests and utilizes 40% of arable land. Furthermore, producing 1 kg of meat from ruminants requires 2.8 kg of feed that is also edible by humans (FAOSTAT, 2018). The livestock sector is also a major contributor to climate change, accounting for 14.5% of anthropogenic greenhouse gas emissions (FAO, 2017). These environmental and resource pressures necessitate a shift toward alternative proteins to ensure a secure and nutritious food supply.

Plant-based proteins are a dominant and promising alternative that can be sustainably integrated into the global food system. The transition to plant proteins is becoming a mandatory strategy to meet international sustainability targets, such as those in the Paris Agreement (Augustin & Cole, 2022). Plant-based meat alternatives require fewer resources, using 47% less land and 72% less water than conventional meat (Good Food Institute, 2019). Moreover, plant protein cultivation

generates fewer greenhouse gases and can improve soil fertility through nitrogen fixation. For example, rotating pulse crops with wheat has been shown to increase wheat yields by 13% (Khakbazan et al., 2018). Among pulses, chickpea (*Cicer arietinum*) is particularly valuable due to its high protein content (18-25%), balanced amino acid profile, and functional versatility. While chickpea protein shows great potential for food applications, its quality and recovery efficiency depend heavily on the extraction method used (Grasso et al., 2022).

Traditional protein extraction techniques include wet methods (e.g., alkaline and salt-based extraction) and dry fractionation. While wet methods can produce high-purity isolates and dry methods are more energy-efficient, both have significant drawbacks (Tang et al., 2024). Dry fractionation, for instance, yields a concentrate with low purity and high antinutritional content, limiting its application. Alkaline extraction, a common wet extraction method, uses harsh chemicals that can denature protein structure, reducing its digestibility and imparting a bitter flavor, thereby compromising its techno-functionality (Perovic and Antov, 2022). As the market for high-protein foods expands, the demand for ingredients with high techno-functionality that do not impair the organoleptic properties of the final product has become critical. This has led to research into novel extraction methods and increasingly, pretreatment techniques. Pretreatment can enhance protein extraction yield and functionality while complementing existing methods. Among these emerging technologies, cold plasma has shown remarkable potential to provide sustainable solutions for the protein industry.

Cold plasma is a partially ionized gas containing a rich mixture of reactive species, including radicals, excited molecules, and ions (Olatunde et al., 2023). Unlike high-temperature or thermal plasmas, cold plasmas exist in a non-equilibrium state where the gas remains at or near room temperature. This non-thermal characteristic makes it ideal for treating heat-sensitive biological materials. Cold plasma technology is efficient and eco-friendly and has been applied in food preservation and agriculture to improve seed germination and sprouting (Asefi & Singh, 2025). Its ability to induce structural modifications in biopolymers, such as proteins, through reactions like oxidation and crosslinking, has made it a subject of growing interest for improving protein functionality. A highly promising application of this technology is Plasma-Activated Water (PAW), which is created by treating water with cold plasma, altering its reactivity and physicochemical properties, including pH, conductivity, and oxidation-reduction potential. PAW offers a more uniform and scalable treatment method than direct plasma application, which can cause surface

etching and degradation of bioactive compounds (Gülenç et al., 2024). While early research on the area focused on modifying the properties of finished protein isolates, recent studies have explored plasma as a pretreatment to improve extraction efficiency. Studies on soybean (Anbarasan et al., 2022), *Chlorella vulgaris* (Zocher et al., 2019), and Camelina (Rezaei et al., 2023) have shown that plasma pretreatment can significantly increase protein yield and enhance functionality. For example, treating sesame cake with plasma increased the protein extraction rate by up to 16.42% (Hou et al., 2025). Despite these promising results, no studies have investigated the use of PAW as a pretreatment in protein extraction. PAW is simple to prepare and can easily be integrated into existing wet extraction workflows, overcoming the uniformity challenges associated with direct plasma treatment.

Therefore, this study aims to investigate the use of Plasma-Activated Water (PAW) as a novel pretreatment in the extraction of chickpea proteins using alkaline, salt-based, and deep eutectic solvent extraction methods. This research assessed how varying plasma conditions (voltage and time) influenced the physicochemical properties of PAW (Conductivity, pH, Oxidation-Reduction Potential) and its impact on the extraction yield of chickpea proteins. Furthermore, it determines the impact of including PAW in various extraction methods on the structural and techno-functional properties of extracted chickpea proteins. The findings are expected to lay the foundation for green, efficient pretreatment that enhances the quality and utility of chickpea protein in the food industry.

4.3. Materials and Methods

4.3.1. Materials

Chickpea seeds (Kabuli variety) were obtained from Avena Foods (Rowatt, Saskatchewan, Canada). Sodium hydroxide, Bradford Serum Albumin Standard, hydrochloric acid, and sodium chloride were supplied by Fisher Scientific (Ottawa, Ontario, Canada). Precast gels, Coomassie blue R250, Protein standards (10-250 kDa), and 2-mercaptoethanol were sourced from Bio-Rad Laboratories (Mississauga, Ontario, Canada). All these reagents were purchased in analytical grade.

4.3.2 Experimental Setup

In this study, a dielectric barrier discharge system (Advanced Plasma Solutions, Malvern, USA) was employed to generate cold plasma for the production of the PAW. A full factorial design was

applied to optimize the processing conditions for PAW preparation, with two factors: applied voltage (kV) and treatment time (minutes), at the levels specified in Table 13 below. This experimental design resulted in 14 treatment combinations, each conducted in triplicate, yielding a total of 42 experimental runs. High-purity argon gas (99.9%) was used as the working gas at a constant flow rate of 3 L/min. The response variables evaluated to characterize PAW and its extraction performance included conductivity (μS), pH, ORP, and Protein Yield (mg/g).

Table 13: Independent variables of PAW at five levels used in the multi-level factorial design

Independent Variables	Levels			
	1	2	3	4
Voltage (kV)	10	15	20	25
Time (min)	5	10	15	20

4.3.3. PAW preparation and chickpea treatment - optimization

100 ml of distilled water was treated with the DBD plasma system under the predetermined experimental conditions. After PAW preparation, 1g of defatted flour was dispersed into 10 ml of PAW in 50 ml Falcon tubes, and the mixture was vortexed (10-15 seconds), then homogenized at 20,000 rpm for 2 minutes. The contents were stirred in the PAW solution for 10 minutes at 200 rpm and centrifuged (12000 rpm, 10 minutes, 4 °C). Protein content (mg/g) was quantified in the supernatant using Bradford Assay using UV-spectrophotometer (595 nm) (Genesys 180 UV-Vis Spectrophotometer, Fisher Scientific, Ottawa, ON, Canada). The best condition was selected using the response optimizer function within the factorial design framework in Minitab software (version 21.4.2) based on the composite desirability index. Conductivity was determined using an Orion Star conductivity meter (Water Analysis Instruments, MA, USA). The pH and oxidation-reduction potential were determined using a pH meter (Thermofisher Scientific, MA, USA).

4.3.4. Protein extraction and protein yield

Using the selected PAW condition, three protein extractions, Alkaline (ALK), Monovalent salt (MSE), and Deep eutectic solvent (DES) extraction were performed. PAW replaced distilled water in these three extractions, creating a modified extraction method (ALK-PAW, MSE-PAW, DES-PAW). Meanwhile, control experiments were conducted using distilled water as the extraction

solvent (ALK-C, MSE-C, DES-C). Finally, protein isolates were also produced using only PAW without the use of any extraction method (PAW).

4.3.4.1 Alkaline extraction

The method used was adapted from a protocol described by Karaca, Low & Nickerson (2011) with some modifications. Defatted chickpea flour was dissolved in water in a ratio of 1:10 (w/v), after which homogenization (20,000 rpm for 2 minutes) was done. The pH was then adjusted to 9.0 using 5 M NaOH, and the extraction was performed for 2 hours at 23 °C while continuously stirring at 200 rpm. The resulting solution was then centrifuged (10,000 rpm for 20 minutes at 4 °C), and the supernatant was collected. The pH of the recovered supernatant was adjusted to 4.5 with 2 M HCl, and the mixture was allowed to precipitate overnight at 4 °C. The precipitate was recovered and washed twice by centrifugation (10,000 rpm for 20 minutes, 4 °C), after which it was dialyzed using 3.5 kDa molecular weight cut off (MWCO tubing) for 120 hours at 4 °C. The dialyzed solution was then lyophilized and stored at -20 °C.

4.3.4.2 Monovalent salt extraction

Salt extraction was performed using a method described by Karaca, Low & Nickerson (2011) with modifications. Chickpea flour was dispersed in NaCl solution (0.8 M) at a ratio of 1:10 (w/v). The dispersion was then homogenized (20,000 rpm for 2 minutes), followed by extraction for 1.5 hours at 23 °C, with constant stirring at 200 rpm. The mixture was then centrifuged to remove by-products, after which the supernatant was dialyzed using a 3.5 kDa (MWCO) dialysis tubing for 120 hours at 4 °C. The dialyzed solution was then freeze-dried and stored in airtight bags at -20 °C.

4.3.4.3 Deep eutectic solvent-based extraction

The protein extraction was performed using the method described by Hewage et al. (2024) with minor modifications. Defatted chickpea flour was added to the prepared DES solution (1:15 w/v), consisting of a mixture of choline chloride and glycerol in a 1:2 molar ratio, with 70% water substitution. The solution was then homogenized (20,000 rpm, 2 minutes), and extraction was performed in a circulating water bath for 2 hours at 50 °C, with stirring at 200 rpm. Centrifugation (10,000 rpm, 20 minutes, 4 °C) was performed to remove precipitates, and the supernatant was

dialyzed using a 3.5 kDa MWCO dialysis tubing for 120 hours. The dialyzed solution was then freeze-dried and stored at -20 °C.

4.3.5.4 PAW-based extraction

Defatted chickpea flour was dispersed in PAW in a ratio of 1:10 (w/v) and homogenized (20,000 rpm for 2 minutes). Extraction was then performed for 2 hours at 23 °C, with constant stirring at 200 rpm. The mixture was then centrifuged (10,000 rpm, 20 minutes, 4 °C) to remove the precipitates. The supernatant was also dialyzed using a 3.5 kDa MWCO dialysis tubing for 120 hours, and the resulting solution was freeze-dried, after which it was stored at -20 °C for future analysis. All extracted proteins were assessed for protein purity, recovery rate, and protein yield using the following equations

$$Protein\ Yield\ (\%)DW = Wp \times \frac{P}{Pr} \times 100 \quad (1)$$

$$Protein\ Recovery\ Rate\ (\%) DW = \frac{Wp \times P}{Wr} \times 100 \quad (2)$$

Wp = Weight (g) of protein isolate (DW)

P = Protein content of protein isolate using the Dumas method(DW)

Pr= Protein content of defatted chickpea flour (DW)

Wr= Weight of initial defatted chickpea flour on DW basis

4.3.6 Proximate analysis

The moisture content of extracted protein isolates was determined by drying samples at 105 °C in an air oven (Thermo Fisher Scientific, Pittsburgh, PA, USA) overnight, then weighing at 6-hour intervals until a stable weight was obtained. Crude protein content was obtained using the Dumas method with a Nitrogen Analyzer (Rapid N exceed, Elementar, Langenselbold, Germany). The ash content was also measured by combusting samples at 550 °C overnight in a box furnace (Model 1100, Thermo Scientific, NC, USA). Crude fat content was evaluated using the Buchi Fat Extractor E-500. All experiments were performed in triplicate to ensure the accuracy and reliability of the results.

4.3.7. Fourier Transform Infrared Spectroscopy (FTIR)

The secondary structure of the chickpea protein isolates was determined using an Invenio FTIR spectrometer (Bruker, Billerica, MA, USA). Scans at a resolution 4 cm^{-1} using 120 scans across a range of $400\text{--}4000\text{ cm}^{-1}$. Prior to the analysis, all spectra were baseline-corrected and normalized to minimize spectral variations due to sample concentration and path length. Amide I peak ($1600\text{--}1700\text{ cm}^{-1}$) which is primarily associated with the C=O stretching vibration of peptide bonds and is highly sensitive to protein structure, was selected for detailed analysis. Second derivative spectra were calculated to enhance the resolution of overlapping component bands and to facilitate accurate peak identification. Curve fitting of the amide I was performed using Gaussian peak functions in Origin 2024 software (OriginLab Corporation, MA, USA).

4.3.8 Morphological properties

The surface morphological properties of the protein isolates were evaluated using a scanning electron microscope (Quanta 640 FEG, FEI Company, OR, USA) equipped with an Everhart-Thornley (ET) detector. Prior to this, the samples were coated with a gold palladium layer using a sputter coating unit and dried under nitrogen purging. The microscopic images were analyzed using xT microscope control software (Quanta 650 FEG, FEI Company, OR, USA).

4.3.7.3 SDS-PAGE analysis

SDS-PAGE was performed to analyze the polypeptide composition of the extracted protein isolates, as described by Hewage et al. (2024), with some modifications. Protein solutions (4 mg/ml) were prepared in 5% SDS solution and heated at $85\text{ }^{\circ}\text{C}$ for an hour. Afterwards, $100\text{ }\mu\text{l}$ of each sample was added to $95\text{ }\mu\text{l}$ Laemmli buffer for reducing conditions and to $100\text{ }\mu\text{l}$ Laemmli buffer for non-reducing conditions, respectively. For the reducing condition, $5\text{ }\mu\text{L}$ of 2-mercaptoethanol was added to the sample buffer, and all samples were heated at $90\text{ }^{\circ}\text{C}$ for 5 minutes. The prepared samples, along with a Precision Plus Protein Standard, were loaded onto a 4-15% Mini Protein TGX precast gel. Electrophoresis was performed at 150V for 1 hour using a Mini-Protean II electrophoresis cell. The gels were stained with Coomassie Brilliant Blue R-250 for 1 hour, then destained with a 50% methanol/10% acetic acid solution in water for 2 hours. Finally, the resulting protein bands were imaged using the Bio-Rad ChemiDoc[™] imaging system (Bio-Rad Laboratories, CA, USA).

4.3.7.4 Particle size distribution

A static laser diffraction technique was used to measure the particle size distributions of chickpea flour and protein isolates, following the protocol of Alonso-Miravalles et al. (2019). Using the Mastersizer 3000 system (Malvern Instruments Ltd, Malvern, UK), a 1% protein suspension was prepared in distilled water at pH 7 and stirred overnight at 4 °C. The suspensions were introduced to the dispersion unit, using distilled water as the medium. The refractive and absorptive indices for chickpea proteins were set at 1.45 and 0.1, respectively, while the dispersant refractive index was 1.33. The obscuration level was adjusted to approximately 6%. Particle size distribution was expressed using volume-weighted mean diameter (D_{4,3}), surface-area weighted mean diameter (D_{3,2}), and volume percentiles (D_v 0.1, 0.5, and 0.9).

4.3.7.5 Surface hydrophobicity

Surface hydrophobicity was evaluated based on the method described by Chukwuejim & Aluko (2025). Chickpea protein samples were initially dissolved in 0.1 M sodium phosphate buffer (pH 7.0) and then diluted to concentrations of 0.005%-0.025% (w/v). For each diluted protein solution, 20 µL of 1-anilino-8-naphthalenesulfonate (ANS; 8 mM in phosphate buffer) was added to 4 mL of the diluted protein solution. The fluorescence intensity (FI) was measured using a Varioskan Lux Microplate Top Absorbance Reader with excitation at 390 nm and emission at 470 nm. A linear regression analysis was performed on the FI-protein concentration plot, and the slope of the line was used to quantify surface hydrophobicity.

4.3.7.6 Color analysis

L*, a*, and b* values of the chickpea samples were determined using a color spectrophotometer Protein samples were (CM-3500 d, Minolta Co. Ltd, Osaka, Japan). Prepared protein samples were placed in a specialized glass cell to provide a level surface and the color values were recorded in triplicates.

4.3.9. Solubility

Protein solubility was determined according to a protocol described by Higa, House & Nickerson (2024) with some modifications. A 0.5% protein solution was prepared in distilled water and hydrated overnight at 4 °C. The suspension was then stirred for 1.5 hours at 23 °C, and the pH of the solutions was adjusted to 3, 5, 7, and 9 using 1 M NaOH and 1 M HCl. Afterwards, the solution

was stirred for 1.5 hours at 200 rpm (23 °C), after which centrifugation was performed at 12,000 rpm for 10 minutes (4 °C). The soluble protein content was determined using the Bradford assay with bovine serum albumin as the standard. The protein solubility was expressed as the amount of soluble protein in the supernatant per gram of total protein in the initial sample.

4.3.9.2 Water and Oil Holding capacity

The water- and oil-holding capacity was determined using a method described by Tontul et al. (2018) with some modifications. For the WHC, 0.15 g of sample was dispersed in 1.5 mL of water in a pre-weighed 2 mL Eppendorf tube. The mixture was then vortexed for 1 minute and allowed to stand for 30 minutes at 23 °C, after which it was centrifuged at 7000 g for 30 minutes. The supernatant was decanted and excess water drained for 15 minutes. Then the tubes were reweighed. For the OHC, 0.17 g of sample was dispersed in 1.67 ml of oil and followed the same procedure as for the WHC. The WHC and OHC were calculated using the following equation

$$\text{WHC or OHC\%} = \frac{\text{Weight of the water or oil absorbed by sample}}{\text{Weight of the initial sample}} \times 100 \quad (4)$$

4.3.9.3 Emulsification properties

The Emulsion Activity Index (EAI) and Emulsion Stability Index (ESI) were determined using the method described by Karaca et al. (2011). 0.5% protein solution was prepared in the following buffers at different pH, 0.1 M Glycine-HCl buffer (pH 3), 0.1 M Acetate Buffer (pH 5), 0.1 M Phosphate Buffer (pH 7), and 0.1M Tris Buffer (pH 9). This was followed by the addition of 5 ml canola oil, and the mixture was homogenized at 20,000 rpm for 1 min (Model 850, Fisher Scientific, Ottawa, ON, Canada). 50 µL of the emulsion from the bottom of the tube was immediately transferred into 5 mL of 0.1% Sodium dodecyl sulfate (SDS) solution and vortexed for 10 seconds. The absorbance was then determined using a spectrophotometer (Genesys 180 UV-Vis Spectrophotometer, Fisher Scientific, Ottawa, ON, Canada) at 500 nm. After 10 mins, another aliquot (50 µL) was taken from the bottom of the tube, dissolved in 5 mL SDS solution (0.1%), and the absorbance was also read at 500 nm. The EAI and ESI were determined using the following equations

$$\text{EAI (m}^2\text{/g)} = \frac{2 \times 2.303 \times A_1 \times DF}{C \times \varphi \times 10000} \quad (5)$$

$$\text{ESI (min)} = \frac{A_1}{A_1 - A_2} \times t \quad (6)$$

A1 = Absorbance after emulsion, DF = Dilution factor, C = protein weight, φ = Volume of oil in emulsion, A2 = Absorbance of the emulsion after 10 min, t = 10 minutes.

4.3.9.3. Foaming properties

The foaming capacity (FC) and foaming stability (FS) were done using a protocol described Vogelsang-O' et al. (2020) with slight modifications. 1% protein concentration was measured using different buffers: 0.1 M Glycine-HCl (pH 3), 0.1 M Acetate (pH 5), 0.1 M Phosphate (pH 7), and 0.1 M Tris (pH 9). The protein solution was vortexed (30 seconds) and homogenized (20,000 rpm for 2 minutes) using a 20 mm shaft probe. After 10 seconds, the initial foam volume (V_o) was recorded and again measured after 30 minutes (V_1). FC and FS were determined using the following equations.

$$FC\% = \left(\frac{V_o}{20ml}\right) \times 100 \quad (7)$$

$$FS\% = \left(\frac{V_1}{V_o}\right) \times 100 \quad (8)$$

4.3.9.4 Least gelling capacity (%)

The Least Gelling Capacity was determined using a method described by Vogelsang-O' et al. (2020). Protein dispersions were prepared by mixing various concentrations of protein (1-20% w/v) with 1ml of 0.1M buffer solutions at different pH levels (pH 3, 5, 7, and 9). Each sample was vortexed thoroughly for 1 min and then hydrated at 4 °C for 16 hours. The protein dispersions were heated in a water bath at 95 °C for 1 hour, cooled under running cold tap water, and stored at 4 °C for 16 hours to allow gel formation. Afterwards, tubes were inverted to observe gelation behavior. The Least Gelling Capacity was defined as the lowest protein concentration at which the sample did not flow upon inversion of the tube. The LGC value was recorded for each pH condition accordingly.

4.3.9.5 Statistical Analysis

Experimental data were analyzed using MINITAB software (2025 version, Minitab LLC, PA, USA). A one-way ANOVA with post-hoc Tukey (LSD) test was done to compare means between specific groups. A two-way ANOVA was subsequently used to identify the significant factors (Method of extraction, PAW inclusion, and their interactions) influencing the measured responses.

Graphing and data visualization were performed using Origin 2024 (OriginLab Corporation, MA, USA).

4.4 Results and Discussion

4.4.1. Optimization of plasma conditions for maximum protein yield

The protein yield, influenced by the different applied voltages (kV) and treatment times, is presented in Table 14.0 below. Protein yield ranged from 159.61 ± 6.87 mg/g to 173.17 ± 6.26 mg/g DW, demonstrating that PAW processing conditions significantly influence protein extraction efficiency. These variations reflect changes in PAW chemistry, particularly alterations in reactive oxygen and nitrogen species (RONS) concentration, pH, and oxidation-reduction potential, which collectively govern PAW-protein interactions. Several studies have reported that RONS generated during plasma activation can induce oxidative modifications in proteins, including oxidation of -N-H, -C-H bonds, thereby altering protein solubility and extractability (Jiang et al., 2020). In the present study, the lowest protein yield (159.61 ± 6.87 mg/g DW) was obtained at 20 kV and 20 minutes, whereas the highest yield (173.17 ± 6.26 mg/g) was achieved at 20 kV and 5 minutes. This trend suggests that mild plasma activation conditions enhance protein solubilization, while prolonged exposure promotes protein denaturation and aggregation, ultimately reducing extractable protein.

PAW-induced structural modifications may either partially unfold proteins, generate smaller peptides, or ionize amino and carboxyl groups, thereby increasing protein-water interactions and solubility (Olatunde et al., 2023). However, excessive plasma exposure can lead to extensive protein denaturation and aggregation, resulting in decreased solubility. This effect was also reported by Asefi et al., who found that a 1-minute plasma treatment produced the highest protein solubility (30 g/100 g DW), while extending the treatment time to 15 minutes reduced solubility to 24 g/100 g DW. This behavior underscores the importance of defining an optimal plasma treatment window to balance structural modification with oxidative stability.

The Pareto chart (Fig. 11a) shows the factors that significantly influenced protein yield. Treatment time emerged as the only statistically significant factor, while voltage and the interaction between voltage and time exerted negligible effects. This observation can be attributed to the fundamental mechanism of PAW chemistry, which is primarily governed by the accumulation of long-lived RONS in the aqueous phase. Longer treatment time allows the progressive accumulation of species

such as hydrogen peroxide, nitrite, and nitrate, leading to a more acidic, oxidative environment. Meanwhile, increasing the voltage typically increases the intensity of the plasma discharge and the rate at which RONS are initially formed. Therefore, higher voltages accelerate RONS production, but if the water quickly becomes saturated or the highly reactive species recombine into more stable forms, the marginal benefit of increased voltage diminishes. Zhou et al. (2018) observed a saturation effect, in which the concentrations of ROS and RNS increased linearly for up to 20 minutes of treatment, attributed to recombination processes involving the high concentration of H₂O₂ in a smaller volume. This suggests that, to optimize protein yield with PAW, researchers should focus on treatment time rather than fine-tuning voltage settings. The main effect plot in 13b shows a sharp drop in protein yield as the treatment time increased from 5 minutes to 10 minutes, and then to 20 minutes. Meanwhile, the voltage curve remained relatively stable at lower voltages before declining at 25 kV.

Table 14. Protein yield (mg/g) obtained from different PAW conditions

Run Order	Voltage (kV)	Time (min)	Protein Yield (mg/g)
1	10	10	170.19±4.91
2	25	20	165.10±3.04
3	10	5	169.12±4.28
4	15	5	172.03±6.06
5	25	15	163.48±10.44
6	25	10	165.25±2.06
7	20	10	165.85±7.65
8	20	5	173.17±6.26
9	20	15	170.77±5.79
10	15	15	170.01±3.61
11	15	10	162.20±10.96
12	15	20	166.41±5.43
13	10	20	161.67±7.11

14	20	20	159.61±6.87
15	10	15	169.78±6.15
16	25	5	170.55±7.92

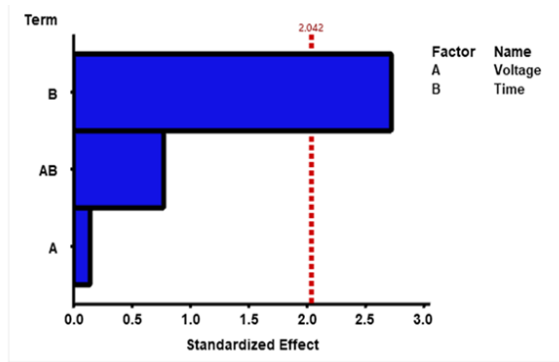


Fig 11a. Pareto Chart of Standardized Effects for Protein Yield

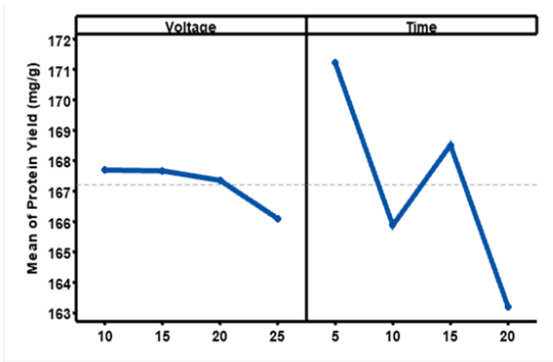


Fig 11b. Main Effect Plot for Protein Yield

Using the response optimizer, 20 kV and 5 minutes were selected as the best conditions for producing plasma-activated water to enhance protein yield (mg/g), with a composite desirability of 0.79. Therefore, mild conditions are sufficient to maximize the potential for protein extraction in the prepared plasma-activated water. A similar trend was reported by Punthi et al. (2023), who optimized PAW (Power = 300-700 W; Time = 30-150 seconds) for the extraction of *Pleurotus ostreatus* polysaccharides and found that 700 W for 58 seconds yielded the highest extraction efficiency.

Table 15. Solution for Optimization

Solution	Voltage (kV)	Time (min)	Protein Yield	Composite Desirability
			(mg/g) Fit	
1	20	5	173.171	0.792063

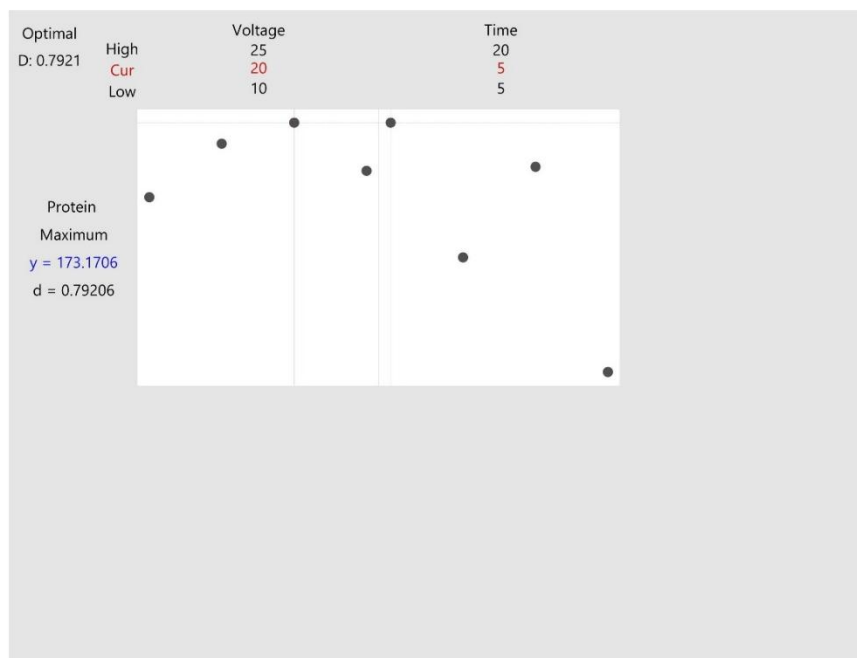


Fig 12: Optimization Plot for Protein Yield

4.4.2 Physicochemical properties of PAW

As reported by Hoeben et al. (2019), electrical conductivity, pH, and the oxidation-reduction potential provide key insights into the chemical activity of plasma-activated water (PAW). Fig. 13 illustrate some notable variations in the physicochemical properties of PAW with changes in voltage and treatment time.

Electrical conductivity is a key indicator of the concentration of reactive species and the ions present in the solution. The Pareto chart (Fig. 13) demonstrates that both voltage and treatment time, as well as their interaction, significantly influenced the changing electrical conductivity of the plasma-activated water. The main-effect plot also shows a linear increase in conductivity with increasing voltage and treatment time. Higher voltage provides increased ionization and excitation energy, which ultimately increases the formation of highly reactive species that diffuse into the water phase, leading to a higher concentration of ions and ultimately increasing conductivity. Similarly, conductivity increased with longer treatment time, owing to the rising concentration of conductive ions in the solution until saturation was reached. Gott, Engeling & Franco (2023) reported similar trends, observing that the water source (tap vs. distilled) and the power supply

(AC & DC) significantly influenced the conductivity of plasma-activated solutions. Notably, distilled water and direct current yielded higher conductivity than tap water and alternating current, highlighting the impact of starting materials and operational conditions on PAW properties. These observations demonstrate the need for further research on the dynamics and mechanisms of RONS across different starting materials (e.g., types of water or other media).

Oxidation-reduction potential (ORP) also increased linearly with voltage and treatment time, ranging from 70.5 ± 7.19 mV to 188.93 ± 3.27 mV. ORP serves as an indicator of electron activity in plasma-activated water, reflecting its oxidative and reductive capacity. It is largely dependent on the concentration and strength of oxidizing species; higher levels of reactive species, such as hydrogen peroxide, increase the oxidative potential. A study by Xu et al. (2016) found that treating distilled water using a premix of argon and oxygen increased the oxidation-reduction potential from 146 mV to 467 mV for 5 to 15 minutes, highlighting the influence of gas composition on reactive species generation. Interestingly, the Pareto chart for ORP shows that the interaction between voltage and treatment time was not significant, unlike conductivity. This suggests that while the total dissolved ion concentration influences conductivity, ORP reflects the oxidizing capacity driven by specific RONS (e.g., H_2O_2 , hydroxyl radicals, and peroxynitrite), whose production rate may respond independently to voltage or time within the tested range.

The pH of PAW decreased from 5.57 ± 0.049 to 3.56 ± 0.06 with increasing voltage and treatment time. This acidification is attributed to the accumulation of reactive species, particularly oxidizing agents like hydrogen peroxide. Lower pH not only affects protein solubility and extraction efficiency but also influences the stability of RONS. Wang & Salvi (2021) observed that reduced pH enhances the stability of hydrogen peroxide in PAW. However, the storage of PAW remains a significant limitation to its broader adoption in the food industry (Zhang et al., 2024). Therefore, future research should focus on strategies to prolong the shelf life and reactivity of PAW water for industrial applications.

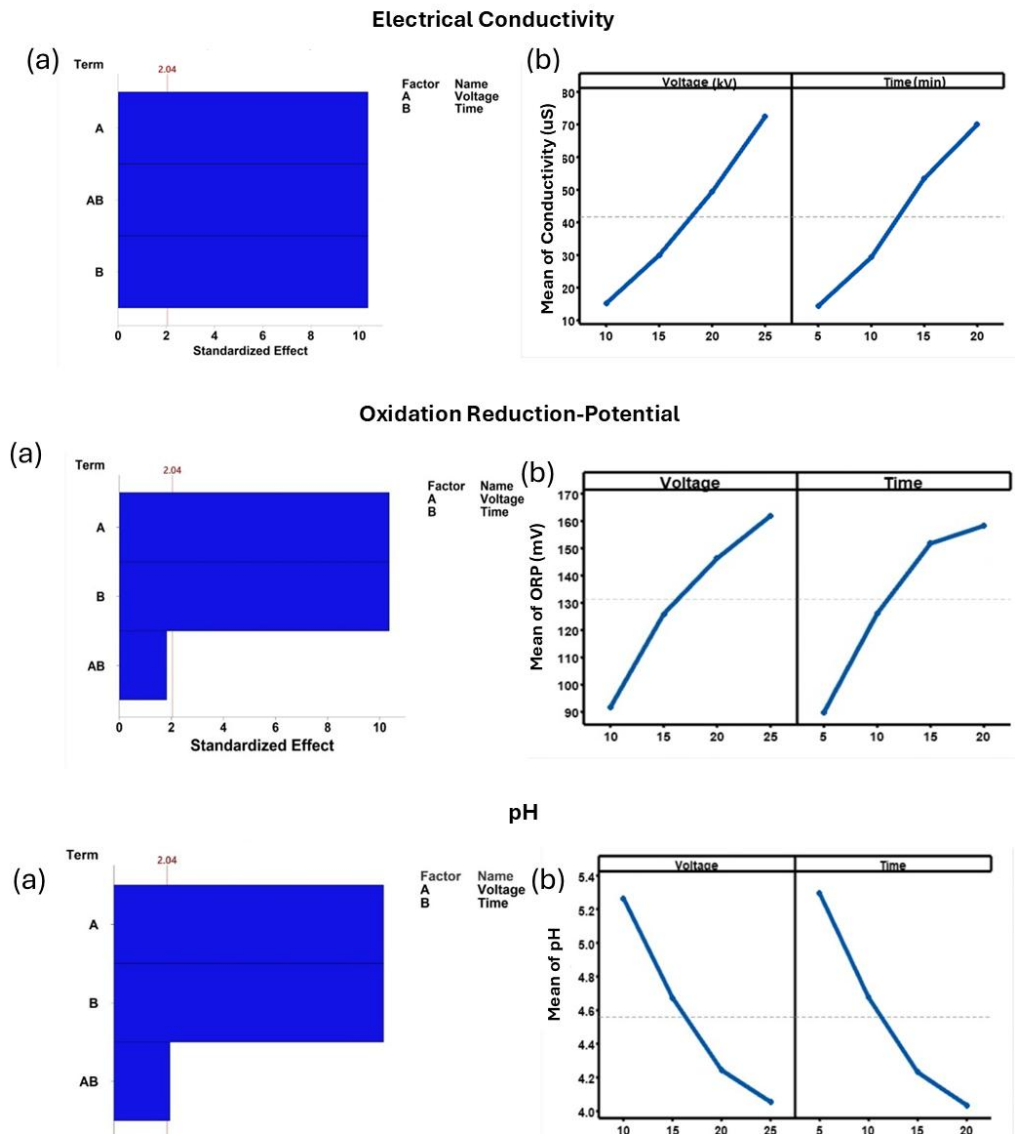


Fig 13. Physicochemical properties of plasma activated water. (a) Pareto chart of standardized effects (b) Main effect plot

4.4.2.3. Correlation: Protein Yield (mg/g), Conductivity (μ S), ORP (mV), pH

The correlation between the physicochemical properties of PAW and protein yield is presented in Table 16 and Fig. 14. Based on the p-value from the pairwise Pearson correlation analysis, all three different water activity metrics, conductivity, oxidation-reduction potential (ORP), and pH, showed a statistically significant relationship with protein yield. A strong negative correlation was observed between pH and both ORP and electrical conductivity. This relationship is expected as the decrease in pH arises from the accumulation of charged ionic species in the water, which simultaneously increases conductivity and oxidative capacity. In contrast, ORP and conductivity exhibited a strong positive correlation ($r = 0.805$), confirming that the same plasma-induced processes responsible for ion generation also enhance PAW's oxidizing potential. However, the correlation was not 0.9-1, reinforcing earlier discussion that not all produced reactive species have the same oxidative activity.

The primary objective of this analysis was to elucidate how these physicochemical parameters influence PAW's protein extraction capacity. Although statistically significant, the correlations between conductivity, ORP, and protein yield were weak, indicating that increases in PAW reactivity do not linearly translate into improved protein yield. In fact, owing to increased protein aggregation and denaturation in high-ORP, high-electrical-conductivity-activated water, protein yield is ultimately reduced. While the application of PAW for protein extraction remains relatively novel, similar correlation trends have been reported in PAW-based microbial inactivation studies. Hu et al. (2023) performed a correlation analysis between reactive oxygen and nitrogen species (RONS) concentrations and the inactivation of *Bacillus cereus* endospores, reporting weak positive correlations ($r=0.143 - 0.476$) between RONS levels and reductions in log CFU/ml. These findings indicate that individual RONS such as nitric and nitrous oxides, peroxide ions, and hydrogen peroxide collectively contribute to microbial inactivation rather than exerting a dominant singular effect. One of the key mechanisms of this inactivation is protein oxidation, which causes enzyme inactivation, structural damage, and disruption of the peptidoglycan cell wall, ultimately leading to the destruction of the microbial population. Therefore, while high RONS concentrations are beneficial for microbial inactivation through protein oxidation, the same oxidative mechanisms

negatively impact protein solubility during extraction, as observed in the present study. These findings highlight the importance of tailoring plasma-activated water production conditions to specific applications and of finding a good balance to explore its multifaceted advantages.

Table 16. Pairwise Pearson Correlations between different Factors of PAW

Sample 1	Sample 2	N	Correlation	95% CI for ρ	P-Value
Conductivity (μ S)	Protein Yield (mg/g)	48	-0.308	(-0.544, -0.026)	0.033
ORP	Protein Yield (mg/g)	48	-0.320	(-0.554, -0.039)	0.027
pH	Protein Yield (mg/g)	48	0.338	(0.060, 0.568)	0.019
ORP	Conductivity (μ S)	48	0.805	(0.675, 0.886)	0.000
pH	Conductivity (μ S)	48	-0.827	(-0.900, -0.710)	0.000
pH	ORP	48	-0.978	(-0.988, -0.962)	0.000

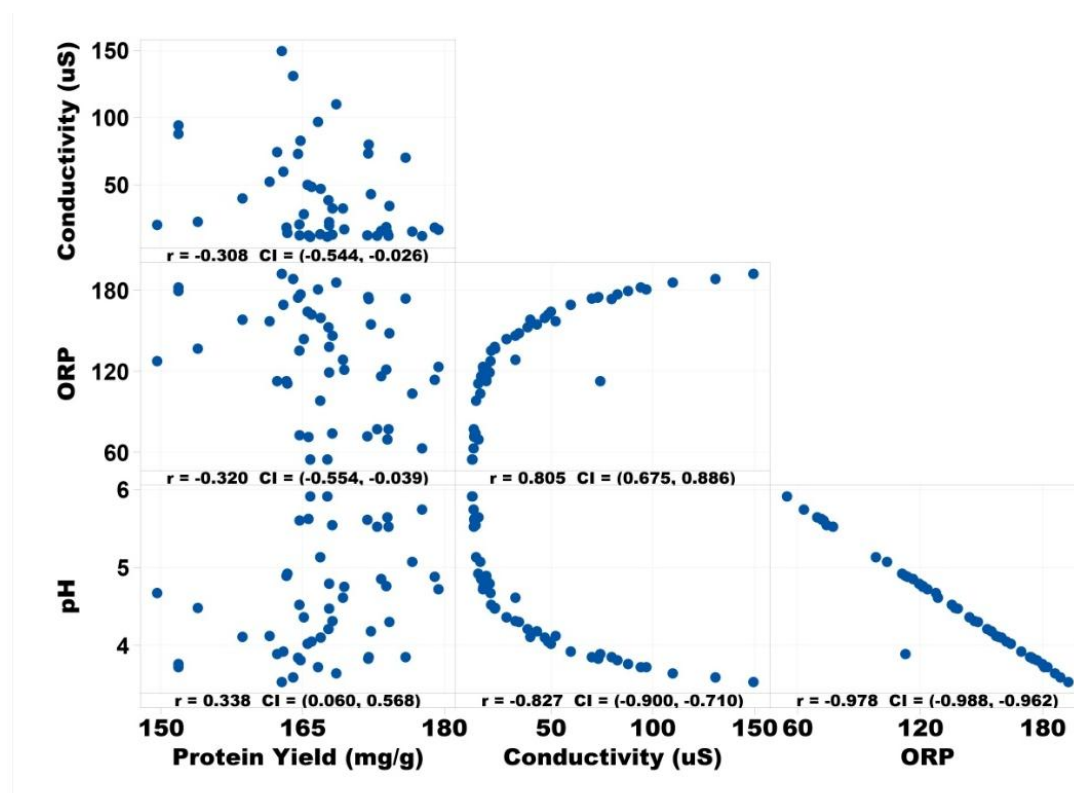


Fig 14. Matrix Plot of Protein Yield, Conductivity, ORP, pH (95% CI for Pearson Correlation)

4.4.3 Protein Extraction Yield

Table 17 summarizes the effectiveness of several extraction methods: Deep Eutectic Solvent (DES-C and DES-PAW), Monovalent Salt Extraction (MSE-C and MSE-PAW), Alkaline Extraction (ALK-C and ALK-PAW), and Plasma Activated Water used as a standalone extraction medium (PAW). The results show apparent differences in how these methods affect the protein purity, recovery rate, and extraction yield.

The standalone PAW method resulted in low protein yield (13.68%) and recovery rate (3.16%), which were significantly lower than those obtained using other extraction methods, where yields ranged from 38-48% and recovery rates from 9-11%. In addition, the protein purity of PAW-extracted protein isolates (76.98%) was significantly lower than that of proteins extracted using DES, MSE, or ALK methods. This outcome can be attributed to the specific role and mechanism of action of plasma-activated water in the chickpea samples. Unlike conventional extraction approaches that primarily rely on pH modulation, ionic strength, and hydrogen bonding to solubilize proteins, PAW primarily facilitates cell wall disruption and structural modification,

thereby improving protein accessibility to the extraction matrix. Previous studies have shown that PAW can modify protein structures and enhance properties such as solubility (Sun et al., 2025). However, in the present study, the moderately acidic pH range (4-5) of the activated water limited protein solubility, resulting in low extraction efficiency. Consequently, these findings indicate that PAW alone is insufficient for efficient protein extraction under the conditions tested, and that combining PAW with established extraction techniques is necessary to achieve higher protein yields.

The other extraction methods (DES, MSE, ALK) achieved high purities (88-89%), recovery rates (9-11%), and yields (38-48%). Results from the control and PAW-modified extractions showed no statistical difference, although DES-PAW showed a slight increase in protein purity (89.14%) compared to the control (87.10%). This suggests that the chemical environment of these extraction systems exerts a buffering effect, influencing the stability and activity of the reactive species and minimizing their impact on the chickpea samples. For instance, alkaline extraction increases the pH of the extraction solvent, which quickly destabilizes the reactive species. A study by Tampieri et al. (2021) showed that the lifetime of a hydroxyl radical in plasma-activated water increased at pH 3 (550 ns) but was significantly reduced at pH 7 (201 ns), indicating reduced oxidative persistence under alkaline conditions. Similar trends were reported by Hou et al. (2025), who applied plasma treatment directly to sesame cake dispersed in water at different voltages. The study found that while the treatments did not significantly affect protein content, the extraction rate increased significantly at 45 kV (16.92%) compared to the control (12.14%), albeit with a concomitant reduction in protein functionality. These discrepancies may be attributed to differences in raw material characteristics (a cake with mostly denatured proteins versus the seeds used in this study) and the mode of plasma application. Further evidence of the benefits of combined approaches was reported by Wang et al. (2024), who observed a significant increase in protein extraction rate when cold plasma treatment was integrated with enzymatic hydrolysis ($83.26 \pm 0.89\%$) compared to enzymatic hydrolysis alone (70.03%). Collectively, these findings highlight the importance of raw material properties, plasma application modes and conditions, processing conditions, and synergistic integration with complementary technologies. Further studies should systematically investigate these factors to optimize cold plasma-assisted protein extraction strategies.

Table 17. Protein Yield, Recovery Rate and Protein Yield of Protein Isolates

Sample	Protein Purity (%)	Recovery Rate (%)	Protein Yield (%)
PAW	76.98±4.10 ^b	3.16±0.59 ^b	13.68±2.58 ^c
DES-C	87.10±1.46 ^a	10.75±0.23 ^a	46.52±0.97 ^{ab}
DES-PAW	89.14±0.10 ^a	10.55±0.49 ^a	45.52±2.24 ^{ab}
MSE-C	89.20±3.21 ^a	9.21±1.24 ^a	38.88±4.29 ^b
MSE-PAW	89.02±1.06 ^a	9.29±0.62 ^a	39.93±2.62 ^b
ALK-C	88.11±3.38 ^a	11.14±0.41 ^a	48.23±1.78 ^a
ALK-PAW	87.11±3.75 ^a	10.25±0.92 ^a	44.35±3.97 ^{ab}

PAW (Plasma-Activated Water-based extraction), DES-C (Deep Eutectic Solvent – Control), DES-PAW (Deep Eutectic Solvent – PAW infused), MSE-C (Monovalent Salt Extraction – Control), MSE-PAW (Monovalent Salt Extraction – PAW infused), ALK-C (Alkaline Extraction – Control), ALK-PAW (Alkaline Extraction – PAW infused). Data was analyzed using ANOVA and Tukey test to determine statistical significance. Different superscript within a column indicate significant difference

4.4.4 Proximate Composition

Table 18 presents the proximate composition of protein isolates obtained using the various extraction methods. The data highlights significant differences in the composition of the extracts depending on the extraction method used.

Protein isolates obtained using plasma-activated water (PAW) alone exhibited significantly higher ash content (3.84%) than those obtained with the other extraction methods. This increase is likely due to the mechanism employed by Plasma Activated Water, which breaks down the cell wall non-selectively, releasing more minerals and inorganic matter that are subsequently co-extracted with proteins. Similar observations have been reported by Jamali et al. (2025), who found that cold plasma treatment of chia seeds increased ash content from 4.12±0.07 in the control to 4.41±0.03 in the treated samples. Likewise, Thirumdas, Deshmukh & Annapure (2016) also found an increase in ash content in basmati rice following cold plasma treatment, although ash levels decreased at higher voltages and longer treatment times. In contrast, the combined extraction methods (DES, MSE, and ALK) resulted in significantly lower ash contents, reflecting their greater selectivity, which favors protein extraction over other components in the sample. Two-way ANOVA performed on the control and PAW-infused extractions revealed that the method employed ($p=0.000$), PAW inclusion ($p=0.016$), and the interaction between the two factors ($p=0.002$) significantly influenced the ash content in the protein isolates. Monovalent salt extraction

significantly reduced the ash content in the PAW-modified isolates compared to the control, indicating higher selectivity for protein over mineral content.

Crude fat content showed notable variation across extraction methods. Similar to the ash content analysis, a two-way analysis of crude fat showed strong evidence that both the type of extraction method ($p=0.000$) and whether PAW was used ($p=0.000$) significantly impacted the fat content of the protein isolate. Crucially, there is also a strong interaction effect between the extraction method and PAW inclusion ($p=0.000$). The highest crude fat content was observed in the MSE-PAW isolate ($3.47\pm0.13\%$), suggesting a synergistic interaction between PAW treatment and monovalent salt extraction. PAW likely induced physical stress and RONS-mediated disruption of the chickpea cellular matrix, including oleosome membranes, thereby releasing entrapped lipids. While PAW alone resulted in low fat extraction due to the poor solubility of lipids in polar media, the monovalent salt environment stabilized proteins in the aqueous phase. In contrast, PAW-induced protein modifications may have exposed hydrophobic regions, promoting the formation of protein-lipid complexes that were subsequently co-extracted. In contrast, alkaline extraction, which operates at high pH, promoted selective protein solubilization with significantly reduced lipid co-extraction. These observations suggest that extraction chemistry plays a critical role in modulating lipid-protein interactions. Further studies are warranted to elucidate how PAW-induced modifications affect the structure, functionality, and nutritional quality of co-extracted lipids.

The protein content was significantly lower in PAW-extracted protein isolates, but similar across the other extraction methods. The low purity aligns with previous findings that PAW, when used alone, is less efficient at selectively extracting intact protein and co-extracts more non-protein material. The extraction method ($p=0.527$), PAW inclusion ($p=0.905$), and their interaction ($p=0.448$) did not significantly affect protein purity in isolates from the modified extraction methods. The protein content obtained in this study is similar to that reported by Xu et al. (2017), who found that chickpea protein content ranged from 79.3% to 85.9% across different samples. Carbohydrate content in this study also showed a similar trend, with PAW isolates having the highest content and similar levels across the other extraction methods used, with no change with PAW infusion.

Table 18. Proximate composition of chickpea protein isolates

Sample	Crude Ash (%)	Crude Fat (%)	Protein Content (%)	Carbohydrates (%)
PAW	3.84±0.01 ^a	0.29±0.02 ^g	76.98±4.10 ^b	18.89±4.09 ^a
DES-C	1.03±0.06 ^e	2.60±0.04 ^b	87.10±1.46 ^a	9.26±1.47 ^b
DES-PAW	1.24±0.02 ^{de}	2.09±0.002 ^c	89.14±0.10 ^a	7.52±0.08 ^b
MSE-C	1.93±0.03 ^b	1.87±0.06 ^d	89.13±3.30 ^a	7.08±3.34 ^b
MSE-PAW	1.34±0.02 ^{de}	3.47±0.13 ^a	89.04±1.05 ^a	6.15±1.15 ^b
ALK-C	1.76±0.25 ^{bc}	1.43±0.07 ^e	88.27±3.09 ^a	8.54±2.87 ^b
ALK-PAW	1.53±0.26 ^{cd}	0.83±0.03 ^f	86.73±3.19 ^a	10.90±3.33 ^b

PAW (Plasma-Activated Water-based extraction), DES-C (Deep Eutectic Solvent – Control), DES-PAW (Deep Eutectic Solvent – PAW infused), MSE-C (Monovalent Salt Extraction – Control), MSE-PAW (Monovalent Salt Extraction – PAW infused), ALK-C (Alkaline Extraction – Control), ALK-PAW (Alkaline Extraction – PAW infused). Data was analyzed using ANOVA and Tukey test and presented in dry basis. Different superscript within a column indicate significant difference

4.4.5 Structural Characterization of Protein Isolates

4.4.5.1 Protein Secondary Structures

Fig. 15 presents Fourier-transform infrared (FTIR) spectra illustrating the effects of different extraction methods on protein conformation. Fig 19 shows the raw, overall spectra for the protein isolates in the range of 4000 to 500 cm^{-1} , with distinct absorption bands in the Amide I (1700 – 1600 cm^{-1}) and Amide II (1600-1500 cm^{-1}) regions. These bands primarily arise from CO stretching vibrations (Amide I) and NH bending coupled with CN stretching vibrations (Amide II) of the peptide backbone. All samples exhibited similar overall spectral profiles, indicating preservation of the fundamental protein backbone across extraction methods. Given the high sensitivity of the Amide I region to protein secondary structure, further spectral deconvolution and curve fitting were performed, as shown in panels (b-h). This analysis enabled the resolution of individual peaks corresponding to β -sheets, α -helices, β -turns, and unordered structures, providing insights into extraction-induced conformational changes.

Quantitative analysis of the secondary structure composition within the Amide I region is summarized in Tables S4.3 and S4.4. Across all methods, β -sheets were the dominant secondary structure, accounting for approximately 59-64% of the total structure, which is characteristic of legume proteins such as chickpea. Similar β -sheet content (60.4%) has been reported for chickpea protein isolates by Xu et al. (2020). The predominance of beta sheets is correlated with a more

rigid, ordered structure that significantly impacts protein digestibility, thermal stability, and functional properties. PAW treatment did not significantly influence beta sheet content, though there was a slight increase in beta sheet content for deep eutectic solvent- and monovalent salt-based protein isolates. Two-way ANOVA also confirmed that the extraction method ($p=0.076$), PAW inclusion ($p=0.726$), and their interaction ($p=0.191$) did not have a significant impact on the beta sheet content of the protein isolates. FTIR analysis further distinguishes between intramolecular β -sheet and intermolecular aggregation-prone β -sheets, providing insight into protein aggregation behavior (Tintor et al., 2024). While intramolecular beta sheets are the native and inherent protein structure, aggregation-prone beta sheets are intermolecular structures that form between different protein molecules, usually triggered by processing.

In the present study, PAW-incorporated extractions (DES-PAW and MSE-PAW) showed significantly higher levels of aggregation-prone beta sheets (high-frequency bands), supporting the role of reactive species in promoting protein aggregation. However, this increase in aggregation beta sheets is not only influenced by the inclusion of PAW in the extraction methods but also by the extraction methods themselves. Two-way ANOVA showed that both the extraction method ($p=0.000$) and PAW inclusion ($p=0.000$) significantly influenced beta sheet aggregation, though their interaction was insignificant ($p=0.470$). DES favored higher-aggregation-prone β -sheets in low-frequency bands, whereas alkaline extraction favored higher-aggregation-prone β -sheets in high-frequency bands.

PAW-extracted protein isolates had significantly higher alpha helix content than the other extraction methods, which corresponded to a significant reduction in unordered helices in PAW-PI. This suggests that PAW alone induced fewer conformational disruptions in α -helical regions than the chemically intensive extraction methods. In contrast, all PAW-assisted extraction methods showed significantly higher β -turn content than their non-PAW counterparts. This increase is attributed to RONS-mediated unfolding and structural rearrangement, which enhances protein flexibility and may positively influence functional performance. These conformational changes are consistent with findings reported by Hou et al. (2025), who observed that cold plasma pretreatment during alkaline extraction increased the β -sheet content (from 34.59% to 35.92%), reduced unordered structures (16.24 to 12.04%), and increased the β -turn content (35.54% to 38.23%). The results demonstrate that PAW induces subtle but meaningful secondary structural

modifications, particularly promoting the formation of aggregation-prone β -sheets and β -turns, depending on the extraction environment.

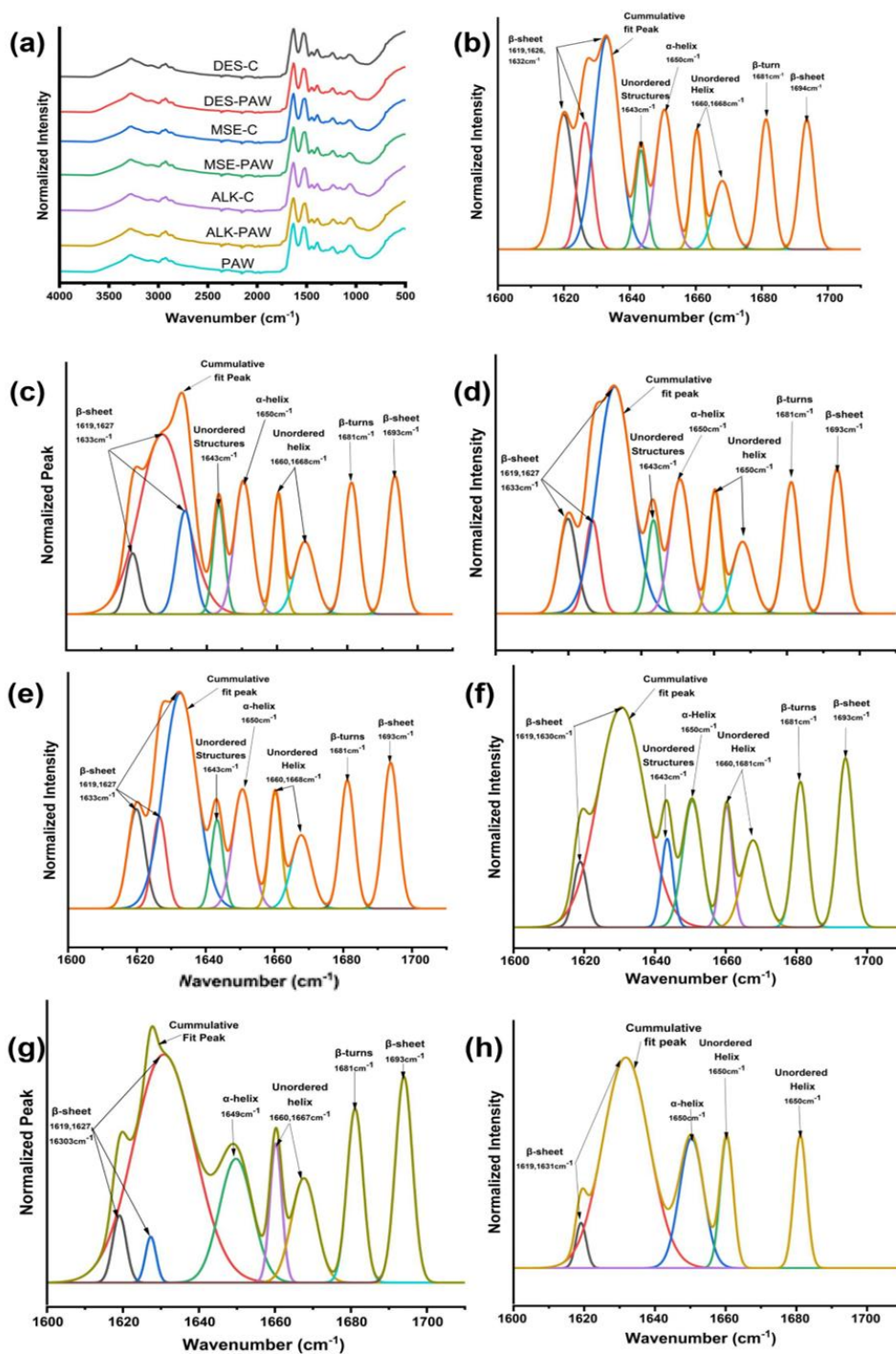


Fig 15. FTIR spectra for chickpea protein fractions (a) FTIR spectra for all chickpea fractions, (b-g) peak fitting of amide I with the different secondary structures (b) Deep-Eutectic Solvent-protein Isolate (Control) (c) Deep-Eutectic Solvent – protein isolates (PAW) (d) Monovalent salt – protein isolate (Control) (e) Monovalent salt – protein isolate (PAW) (f) Alkaline protein isolate (Control) (g) Alkaline protein isolate (PAW) (h) Plasma Activated Water – protein isolate

4.4.5.1 Morphological Properties

Fig. 16 displays the Scanning Electron Microscope (SEM) images of the different protein samples. The SEM images compare the surface morphology and particle characteristics of the protein isolates at three distinct magnifications (500, 1000, and 5000). At $\times 500$ magnification, the protein samples exhibited irregularly shaped, heterogeneous particles. Paw-modified extraction methods, particularly DES-PAW, produced notably larger agglomerates than their respective control samples. Similar trends were observed for ALK-PAW relative to ALK-Control. These morphological observations corroborate the FTIR results, which indicated increased formation of aggregation-prone β -sheets, leading to the development of larger protein aggregates. Supporting this mechanism, Wang et al. (2025) reported that PAW treatment increased the diameter of myofibrillar proteins, as observed using atomic force microscopy, due to the formation of chain-like aggregates.

Furthermore, SEM micrographs at $\times 1000$ magnification revealed a consistently rough, irregular surface morphology across all samples. This roughness is advantageous because it increases the available surface area for interaction with surrounding media. An expanded surface area can enhance key functional properties, such as adsorption capacity, hydration, and solubility. At $\times 5000$ magnification level, more pronounced structural differences were evident. While control samples (DES-Control, Alk-Control, and MSE-Control) exhibited some porosity, PAW-incorporated extraction methods, especially MSE-PAW, showed more well-defined pores and a less compact structure. These features indicate a more open protein matrix, likely resulting from RONS-induced structural modifications. These findings agree with those of Hou et al. (2025), who reported that cold-plasma-treated proteins exhibited more diffuse structures with larger cavities. Notably, the direct plasma treatment applied in their study resulted in more pronounced surface roughness and pore formation than observed in the present study. Variations in voltage, treatment duration, plasma application mode, equipment type, and sample matrix likely contribute to these differences.

Consequently, further investigation is required to elucidate the complex interactions between reactive species and protein matrices and to understand how processing parameters influence protein modification.

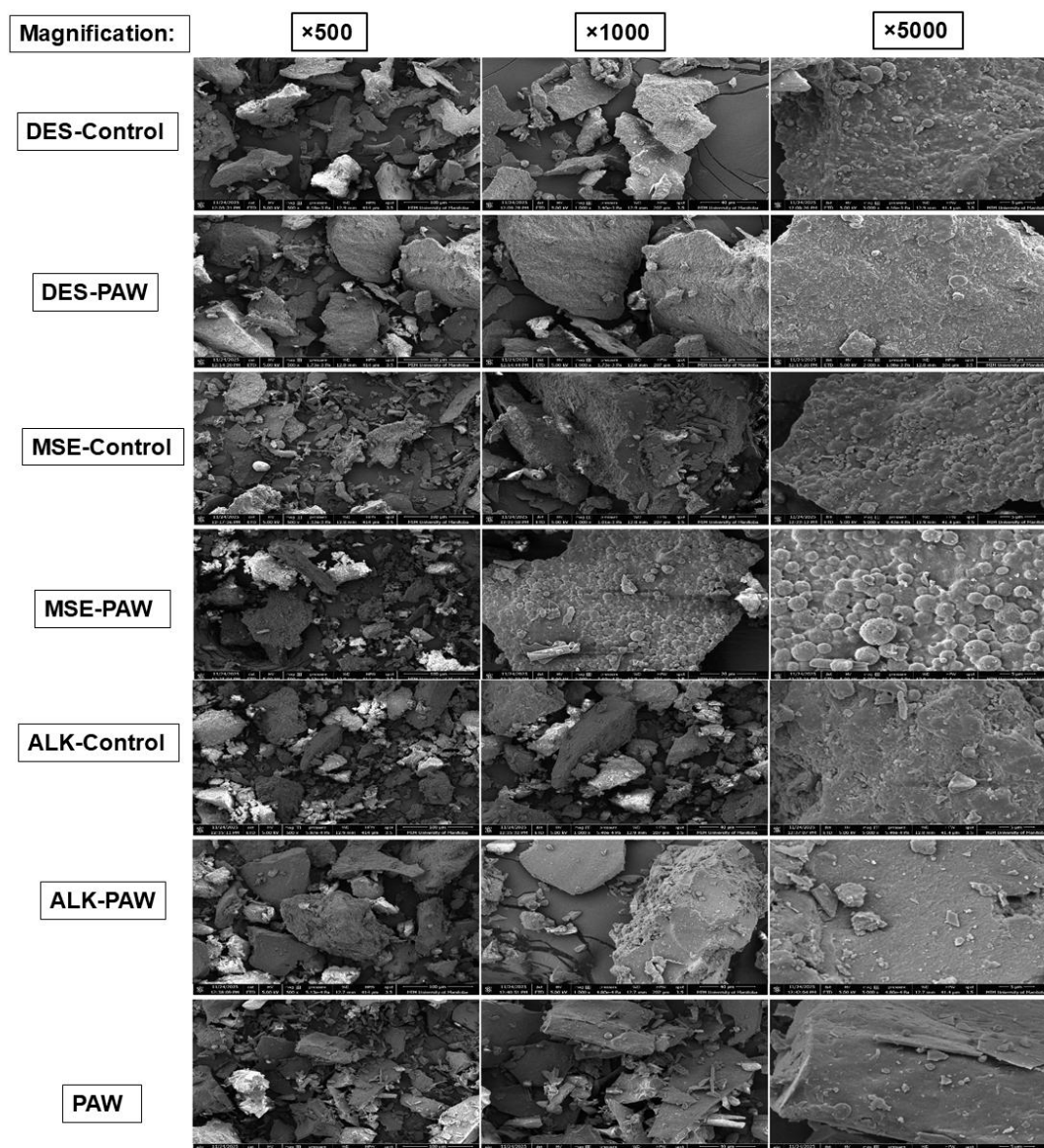


Fig 16. SEM images for chickpea fractions. DES (Control & PAW) – Deep Eutectic Solvent – Protein Isolates (Control without PAW and PAW inclusion respectively); MSE Control – Monovalent Salt Extraction-Protein Isolates (Control without PAW and PAW inclusion respectively); ALK (Control & PAW) – Alkaline extraction – Protein Isolates (Control without

PAW and PAW inclusion respectively); PAW – Plasma Activated Water – Extracted Protein Isolates

4.4.5.2 SDS-PAGE Analysis

Fig. 17 below presents the SDS-PAGE profiles of chickpea protein samples extracted using different methods, analyzed under both non-reducing and reducing conditions. Across all samples, protein bands were observed within the characteristic molecular weight ranges of the major storage proteins, including convicilin, vicilin, and legumin subunits. Overall, the electrophoretic profiles were highly comparable across samples, indicating that neither the extraction method nor the incorporation of plasma-activated water (PAW) substantially altered the protein molecular weight distribution.

Under non-reducing conditions, a prominent band observed at approximately 66 kDa corresponds to the convicilin fraction, while bands in the range of 47-50 kDa are attributed to vicilin subunits. Additional bands around 35-40 kDa represent legumin subunits, which constitute a major portion of chickpea seed storage proteins. The similarity in band intensity and distribution across samples suggests that the native quaternary structures of these proteins were largely preserved regardless of the extraction approach. Upon analysis under reducing conditions, disruption of disulfide bonds led to the dissociation of legumin hexamers into their constituent subunits. This resulted in clearly resolved bands at approximately 35 kDa, corresponding to the acidic subunit, and 19-22 kDa, corresponding to the basic subunit. The appearance of these bands across all samples confirms effective reduction and is consistent with the known molecular structure of legumin proteins. Faint bands at about 75 kDa under reducing conditions were observed in most samples, though they were less pronounced in the deep eutectic solvent control. No unique bands or shifts in molecular weight were observed as a result of PAW incorporation, indicating that the PAW treatment did not induce detectable protein fragmentation or covalent crosslinking by SDS-PAGE. This observation is consistent with findings by Sun et al. (2024), who reported no significant differences in SDS-PAGE profiles between PAW-treated and untreated soy protein isolates.

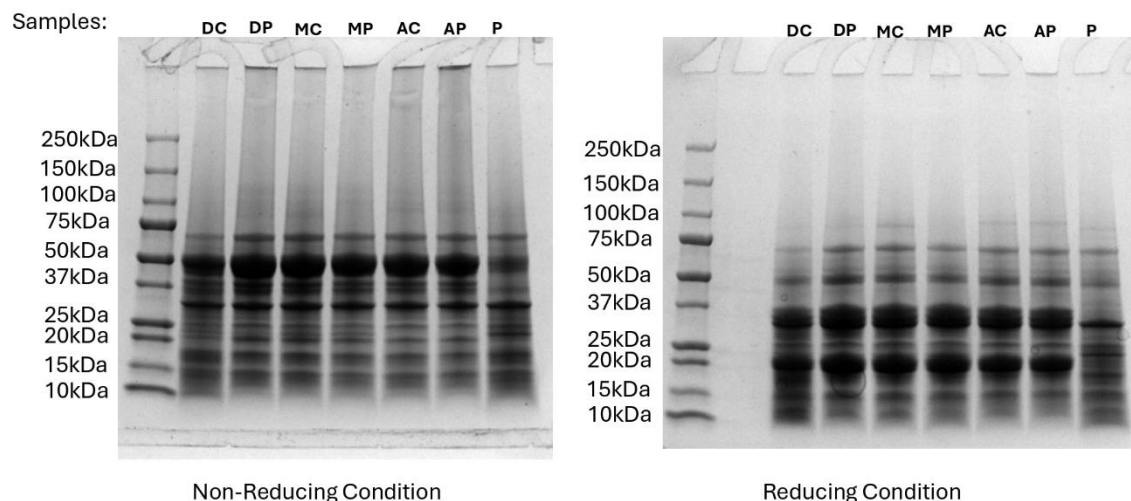


Fig 17: SDS PAGE Profiles of Chickpea Protein Isolates Under Non-Reducing and Reducing Conditions. DC: Deep eutectic solvent-protein isolates (control), DP: Deep eutectic solvent – protein isolates (PAW), MC: Monovalent salt-protein isolates (Control), MP: Monovalent salt-protein isolates (PAW), AC: Alkaline extraction-protein isolates (Control), AP: Alkaline extraction-protein isolates (PAW), P: Plasma Activated Water-Protein Isolates

4.4.5.3 Particle Size Distribution

The particle size distribution metrics of the chickpea protein isolates extracted using different methods are presented in Table 19 and Fig. S4.5. These metrics provide specific insights into the average particle size, the range of particle sizes present, and the distribution shape for each sample. The results clearly indicate that the protein extraction method and the inclusion of PAW in the extractions significantly modified the particle size distribution of the extracted protein isolates. The incorporation of PAW in the extraction generally increased the particle size distribution of the protein isolates. Notably, DES-PAW showed a pronounced increase in the volume weighted mean diameter ($D_{4,3}$) from $35.07 \pm 8.79 \mu\text{m}$ in the control (DES-C) to $73.97 \pm 4.27 \mu\text{m}$. A similar trend was observed for alkaline extraction, where the particle size increased from $24.80 \pm 6.14 \mu\text{m}$ ALK-C to $31.47 \pm 3.55 \mu\text{m}$ (ALK-PAW). These findings align with the FTIR results, which revealed an increase in aggregation-prone β -sheets structures in PAW-incorporated extractions. The interaction between reactive species and protein molecules likely promotes protein-protein interaction and aggregation, leading to the formation of larger particles. The surface-weighted ($D_{3,2}$) mean also showed significant changes. In alkaline extraction, PAW inclusion resulted in a

substantial increase from $5.76 \pm 2.44 \mu\text{m}$ to $14.90 \pm 2.52 \mu\text{m}$, indicating enhanced aggregation at the particle surface level. Two-way ANOVA confirmed that the extraction method ($p=0.034$, 0.000), PAW incorporation ($p=0.027$, 0.004), and their interaction ($p=0.039$, 0.000) significantly influenced the D (3,2) and D (4,3) metrics, respectively, for the different protein isolates. Apart from the increases in particle distributions observed, it was interesting to note that the monovalent salt incorporated with PAW decreased the particle size distribution, although the distributions were statistically similar. It provides deeper insight into how different extraction conditions can modify the activity of reactive species, producing varied properties in the extracted protein isolates.

The particle size percentiles (D_{10} , D_{50} , and D_{90}) further confirmed these trends. Samples such as DES-PAW and PAW-only extracts exhibited larger median and coarse-end particle sizes, indicating broader and coarser distributions. These results are consistent with those of Sun et al. (2025), who reported a substantial increase in soy protein particle diameter following PAW treatment, from $28.86 \pm 2.81 \text{ nm}$ in the control to $280.7 \pm 18.77 \text{ nm}$ after 25 minutes of treatment, attributed to changes in surface hydrophobicity and electrostatic interactions. These structural changes have significant implications for their utilization as functional ingredients in the food industry, as they can influence the texture and stability of food products. While fine particles like MSE-PAW can find applications in beverages (enhanced solubility and dispersibility) and mayonnaise (emulsion and foaming properties), large-sized particles like DES-PAW can be good texturization and gelling agents.

Table 19. Particle Size Distribution of Chickpea Protein Isolates

Particle Size distribution (µm)	DES-C	DES-P	MSE-C	MSE-P	ALK-C	ALK-P	PAW
D [4,3]	35.07±8.79 ^{bcd}	73.97±4.27 ^a	44.93±6.34 ^c	31.03±3.63 ^{cd}	24.80±6.14 ^d	31.47±3.55 ^{cd}	47.1±0.2 ^b
D [3,2]	13.15±6.97 ^{abc}	18.37±0.74 ^b	12.07±2.7 ^{abc}	9.85±1.05 ^{bc}	5.76±2.44 ^c	14.90±2.52 ^{ab}	20.33±1.43 ^a
Dx (90)	71.98±4.9 ^c	173.67±8.50 ^a	110.60±11 ^b	68.70±6.01 ^{cd}	51.70±5.20 ^d	67.67±7.18 ^{cd}	98.4±0.36 ^b
Dx (50)	26.50±8.75 ^{bc}	51.60±5.08 ^a	26.90±7.95 ^{bc}	21.17±2.40 ^c	14.60±5.30 ^c	21.63±4.48 ^c	37.57±0.15 ^{ab}
Dx (10)	5.89±3.06 ^{abc}	8.60±0.51 ^{ab}	6.13±1.37 ^{abc}	4.93±0.67 ^{bc}	2.43±1.66 ^c	6.92±1.12 ^{ab}	9.64±0.18 ^a

4.4.5.3 Surface Hydrophobicity

Changes in the surface hydrophobicity of the protein isolates and the influence of plasma-activated water incorporation are presented in Table 20 below. The surface hydrophobicity (S_o) is a measure of the non-polar surface area of a protein exposed to the aqueous solvent. It is critically linked to functional behaviors such as emulsification, foaming, and gelation. PAW incorporation into the extraction method, as well as PAW used alone, resulted in a significant increase in the surface hydrophobicity of the extracted proteins. For instance, the surface hydrophobicity of DES protein isolates increased from 0.063 ± 0.001 in the control to 0.098 ± 0.002 in the PAW-incorporated samples. Similar findings were made in alkaline extraction, which increased from 0.035 ± 0.013 to 0.055 ± 0.001 , showing a general increase in surface hydrophobicity across extraction methods. Higher S_o values often indicate greater protein unfolding or the presence of hydrophobic amino acids, which can either improve functionality or lead to uncontrolled aggregation. This finding fits with the other results, which observed increased protein aggregation, which can be explained by the increasing surface hydrophobicity that can promote protein agglomeration. This can also be explained by the increasing beta turns observed in the FTIR spectra, which lead to a more loose and unfolded protein structure, increasing the hydrodynamic radius of protein molecules and thereby increasing surface hydrophobicity.

Apart from the extraction-infused methods, PAW extraction alone also had significantly higher surface hydrophobicity, indicating the lone action of the reactive species in improving surface hydrophobicity; however, the different extraction methods also had significant influence on surface hydrophobicity, with monovalent salt having the highest S_o value. According to the ANOVA results, both the extraction method ($p=0.000$) and paw inclusion ($p=0.005$) had a significant influence on surface hydrophobicity, although their interaction ($p=0.094$) was not significant. This observation agrees with findings by Li et al. (2024), who reported that treatment of chicken myofibrillar protein with plasma-activated water significantly increased surface hydrophobicity to 6.49 ug after 12 minutes of treatment. This further highlights the structural modifications that occur in PAW-incorporated protein extracts.

Alkaline extraction-protein isolates (Control), ALK PAW: Alkaline extraction-protein isolates (PAW), PAW: Plasma Activated Water-Protein Isolates

Table 20. Surface Hydrophobicity of the Protein Isolates

Sample	Surface Hydrophobicity (So)
DES-C	0.063±0.001 ^{bc}
DES-P	0.098±0.002 ^a
MSE-C	0.099±0.009 ^a
MSE-P	0.101±0.019 ^a
ALK-C	0.035±0.013 ^c
ALK-P	0.055±0.001 ^{bc}
PAW	0.076±0.011 ^{ab}

PAW (Plasma-Activated Water-based extraction), DES-C (Deep Eutectic Solvent – Control), DES-PAW (Deep Eutectic Solvent – PAW infused), MSE-C (Monovalent Salt Extraction – Control), MSE-PAW (Monovalent Salt Extraction – PAW infused), ALK-C (Alkaline Extraction – Control), ALK-PAW (Alkaline Extraction – PAW infused). Data was analyzed using ANOVA and Tukey test and presented in dry basis. Different superscript within a column indicate significant difference

4.4.5.5 Color

Table 21 shows the color analysis of the protein isolates in the standard CIE L*a*b* color space, which quantifies color objectively and provides crucial information on the aesthetic quality of food products, as color significantly impacts consumer acceptance.

L* value indicates the level of lightness ranging from 0 (black) to 100 (white), meaning that higher values indicate a lighter and brighter sample. The results showed a decrease in lightness with the inclusion of PAW in the extraction methods, though protein isolates from PAW alone had the highest L* value, indicating the highest brightness. These results further highlight the synergistic role of the reactive species and the extraction media in modifying the physicochemical properties of the protein extracts. These observations could be attributed to the action of the reactive species, as confirmed in earlier characterizations. Through its depolymerization and surface-etching reactions, the plant cell wall is degraded, leading to increased extractability of not only proteins but also other components, including pigments, polyphenols, and lipids, which impart color to the final product. Furthermore, the oxidation of the polyphenol components in the sample could lead to a darker coloration, as observed in the study. However, PAW alone creates a polar environment

that is insoluble for these fractions, such as lipids, reducing the co-extraction of these components and thus resulting in a brighter color. However, although selective, the extraction methods also extract these components. For instance, ALK-P had the lowest L* value, which can be attributed to the high pH and PAW, which likely induced browning reactions of the polyphenols and the oxidation of the proteins, darkening the final isolate. This was also backed up with its high b* value, indicating a brown/yellow appearance. Moreover, a* was the highest ALK-PAW and the lowest in PAW.

Light-colored protein isolates like PAW are suited for neutral-colored food products such as yogurt and milk, where minimal interference is desired. In contrast, the brown and yellow products can be applied in applications where color is less critical, such as chocolate-flavored drinks and some meat analogues. Similar findings were made by Segat et al. (2015), where the yellow color parameter (b*) was directly proportional to the duration of the plasma treatment, with increasing yellow color parameter.

Table 21. Color Profile of Extracted Protein Isolates

Sample	L*	a*	b*
DES-C	78.43±0.23 ^b	3.30±0.10 ^c	17.33±0.15 ^c
DES-P	75.70±0.10 ^c	3.83±0.15 ^b	19.70±0.56 ^b
MSE-C	79.87±0.31 ^a	2.70±0.10 ^d	18.40±0.30 ^c
MSE-P	79.27±0.11 ^b	2.57±0.15 ^d	17.90±0.49 ^c
ALK-C	75.23±0.40 ^c	3.50±0.10 ^c	20.73±0.12 ^b
ALK-PAW	69.93±0.68 ^d	4.40±0.10 ^a	21.97±0.67 ^a
PAW	79.17±0.32 ^{ab}	1.87±0.06 ^e	17.53±0.49 ^c

PAW (Plasma-Activated Water-based extraction), DES-C (Deep Eutectic Solvent – Control), DES-PAW (Deep Eutectic Solvent – PAW infused), MSE-C (Monovalent Salt Extraction – Control), MSE-PAW (Monovalent Salt Extraction – PAW infused), ALK-C (Alkaline Extraction – Control), ALK-PAW (Alkaline Extraction – PAW infused). Data was analyzed using ANOVA and Tukey test and presented in dry basis. Different superscript within a column indicate significant difference

4.4.5 Functional Characterization of Protein Isolates

4.4.5.1 Solubility

The solubility profiles of the extracted protein isolates are presented in Fig. 18 below. The solubility varies significantly with extraction method, plasma-activated water (PAW) inclusion, and solution pH. Solubility is a fundamental property that influences other protein functionalities, such as emulsification and foaming.

As expected, solubility profiles of the proteins were at their lowest at pH 5 (0.5 – 9.87%), corresponding to the proximity of this pH to the isoelectric point of the chickpea proteins, where net charge is minimal, and protein aggregation is favored. In contrast, maximum solubility was observed at pH 9 (47-78%), where increased electrostatic repulsion enhances protein dispersion. At pH 3, PAW-modified extraction methods resulted in a reduction in protein solubility for monovalent salt-extracted (MSE-PAW: 43.14%) and alkaline-extracted (ALK-PAW: 29.69%) protein isolates compared to their respective controls (MSE-C: 64.34%; ALK-C: 39.99%). This reduction in solubility can be attributed to PAW-induced protein aggregation, which limits protein-water interactions. In contrast, DES-PAW exhibited greater solubility (61.35%) than DES-C (56.19%), highlighting the synergistic interaction between the deep eutectic solvent and the PAW-derived reactive species, which may promote partial unfolding without excessive aggregation. Beyond PAW incorporation, the extraction technology itself significantly influenced protein solubility. Monovalent salt-extracted protein isolates demonstrated the highest solubility at pH 3 (64.32%), while alkaline-extracted proteins exhibited the lowest solubility (39.99%). This behavior may be attributed to the finer particle-size distribution of monovalent-salt-extracted proteins, which provides a larger surface area for hydration and protein-solvent interactions. Statistical analysis confirmed that the extraction method ($p=0.000$), PAW incorporation ($p=0.000$), and their interaction ($p=0.000$) had significant effects on protein solubility.

At pH 5, all protein isolates exhibited minimal solubility due to aggregation and precipitation associated with near-zero net charge. Notably, PAW-extracted protein isolates displayed significantly higher solubility (9.89%) compared to proteins obtained using other extraction methods (0.58-4.52%). This enhancement suggests PAW-induced conformational modifications that alter surface charge distribution. Mehr & Koocheki (2020) reported that plasma treatment promotes amino acid oxidation, converting positively charged residues into negatively charged ones, thereby increasing their zeta potential and improving protein solubility near the isoelectric

point, findings consistent with those of this study. At pH 7 and 9, solubility trends continued to vary significantly across extraction methods and PAW incorporation. Similar to observations at pH 3, PAW inclusion generally reduced protein solubility compared to the control samples, whereas DES-PAW maintained solubility levels comparable to DES-C. These results provide deeper insight into the role of PAW in chickpea protein extraction, where increased protein aggregation and oxidation, as well as co-extraction of polyphenols and other compounds, may collectively limit protein solubility.

These findings underscore the potential for further optimization through combined pretreatment strategies that balance PAW-induced structural modification with controlled extraction conditions to enhance both solubility and yield. Similar trends were reported by Li et al. (2025), who observed a 16.20% reduction in protein solubility following PAW treatment of pale, soft, and exudative-like chicken protein isolates after 40 seconds, accompanied by increasing turbidity indicative of protein aggregation. Conversely, other studies have demonstrated enhanced protein solubility following plasma treatment (Perinban, Orsat & Raghavan, 2023). Such discrepancies can be attributed to differences in raw material composition, plasma conditions, and treatment modes.

4.4.5.2 Water and Oil Holding Capacity

Fig. 18(b) provides insights into changes in the water- and oil-holding capacities of protein isolates extracted using four extraction methods (DES, ALK, MSE, and PAW) under two conditions: control (without PAW) and PAW-incorporated. The water holding capacity refers to a material's ability to bind with and retain water physicochemically under gravity. It is a vital characteristic that influences the quality, texture, stability, and sensory attributes of food products (Shen et al., 2022). Incorporation of plasma-activated water into the extraction methods significantly increased the water-holding capacity of the extracted protein isolates compared to the controls. ALK-PAW had the highest water-holding capacity (150.52%), which was significantly higher than ALK-C (131.46%). This observation can be attributed to distinct PAW-induced modifications in the protein structure, which expose hydrophilic amino acid residues and increase the hydrophilicity of the protein materials. Furthermore, the increased porosity observed in the SEM images could be a key mechanism for the increased water-holding capacity, as these modified proteins can trap greater amounts of water in this matrix. These findings agree with observations by Acharjee et al. (2023), who reported that plasma treatment of pea protein isolate significantly increased the water-holding

capacity from 2.68 ml/g in the control to 3.16 ml/g after 230 V of plasma treatment for 15 minutes. Enhanced water-holding capacity of protein isolates contributes to juiciness and texture in food applications such as meat analogues and bakery products.

The oil-holding capacity indicates the ability of the proteins to bind and entrap oil molecules, making it pivotal in food applications, as it helps retain fats and oils, which improve the texture and juiciness of food products. The oil-holding capacity was significantly influenced by the different extraction methods ($P=0.004$), whereas incorporation of PAW did not affect the oil-holding capacity of extracted proteins ($P=0.546$). MSE-C and DES-C had the highest oil holding capacity, 159.39% and 149.70% respectively, whereas ALK-C had the lowest oil holding capacity (87.13%). The increase in oil holding capacity is highly influenced by surface hydrophobicity, which increases the ability of proteins to bind to the oil. Therefore, MSE and DES, which had significantly higher surface hydrophobicity, also had higher oil-holding capacity. Although PAW inclusion did not affect the monovalent and deep eutectic solvent-extracted protein isolates, ALK-PAW had a significantly higher oil-holding capacity than the control. This can also be attributed to the increasing surface hydrophobicity resulting from PAW-induced conformational changes in the protein's structure. Liu et al. (2024) found a similar trend, where cold plasma modification of pea protein isolates led to increased oil holding capacity owing to increased exposure of non-polar amino acids and hydrophobic groups, although extended plasma treatment times decreased the OHC. The ability of PAW to improve the water- and oil-holding capacity of alkaline-extracted protein isolates increases their functional value and broadens their applicability across several industrial processes.

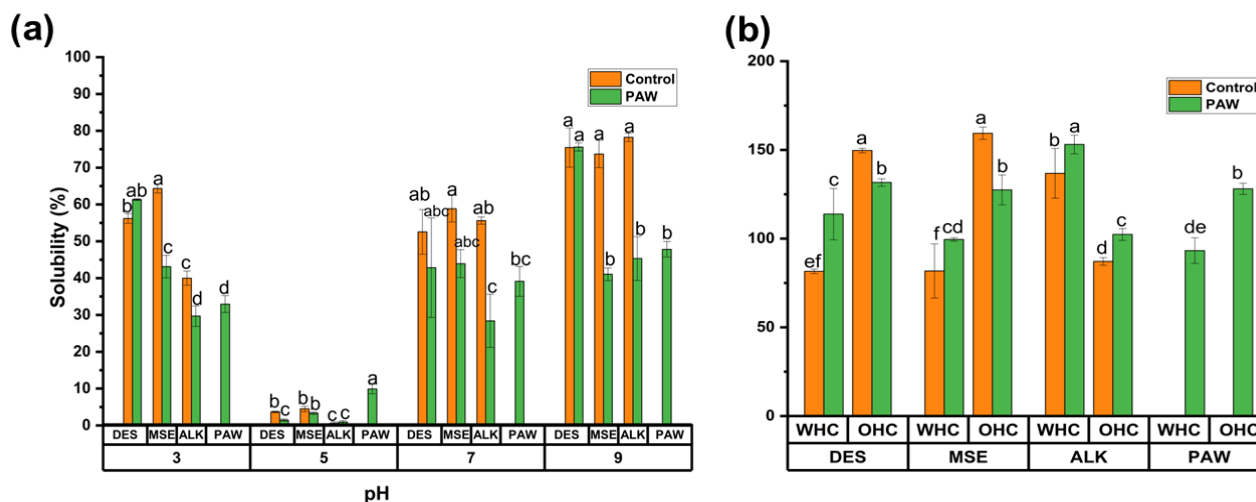


Fig 18: (a) Solubility of Protein Isolates (b) Water Holding Capacity (WHC) and Oil Holding Capacity (OHC) of Protein Isolates. DES C: Deep eutectic solvent-protein isolates (control), DES PAW: Deep eutectic solvent – protein isolates (PAW), MSE C: Monovalent salt-protein isolates (Control), MSE PAW: Monovalent salt-protein isolates (PAW), ALK C: Alkaline extraction-protein isolates (Control), ALK PAW: Alkaline extraction-protein isolates (PAW), PAW: Plasma Activated Water-Protein Isolates

4.4.5.3 Foaming Properties

Fig 19 (a) and (b) illustrate the impact of different extraction methods, PAW incorporation, and pH levels on the foaming properties of the chickpea protein isolates. These protein qualities are instrumental in influencing the lightness, mouthfeel, and texture of aerated products like meringues, whipped cream, and ice cream. The results showed that DES-PAW (143.22%) and MSE-PAW (140.06%) had significantly higher foaming capacity than their controls, DES-C (121.69%) and MSE-C (125.60%), at pH 3. These findings further highlight the functional improvement achieved by incorporating PAW into extraction methods, via PAW-induced modifications to protein structures. This increase in foaming capacity can be attributed to the increasing surface hydrophobicity, which interacts with air bubbles to improve foaming ability. Furthermore, the increased beta turns in the PAW-incorporated protein isolates increase the proteins' flexibility, enabling them to be quickly adsorbed to the air-water interface and thus enabling increased foam formation. At pH5, MSE-PAW had the highest foaming capacity, particularly due to its fine particle size distribution, which increases mobility and diffusivity to the

air-water interface and lowers surface tension. Results from ANOVA indicated that at pH3, pH7 and pH9, the extraction method ($p=0.000$, 0.006 , 0.036 respectively), PAW incorporation ($p=0.010$, 0.000 , 0.036 respectively) and their interactions excluding at pH9, ($P=0.000$, 0.002 , 0.055 respectively) significantly influenced the foaming capacity, however only the extraction method ($p=0.009$) and their interaction ($p=0.039$) were significant at pH5, with PAW incorporation showing an insignificant impact on the foaming properties at pH5. These findings elucidate a pH-dependent response in foaming capacity that influences the impact of PAW-modified protein isolates. Studies on how the modified protein isolate reacts differently under different pH conditions compared to the control can be undertaken. These findings agree with observations made by Perinban, Orsat & Raghavan (2023), who reported that plasma-activated water treatment of whey protein isolate significantly increased the foaming capacity to 225%.

Foaming stability refers to the ability of proteins to maintain foam volume over time. The foaming stability of the proteins was statistically similar at pH 3, showing that neither the different extraction methods nor the incorporation of PAW influenced changes in foaming stability at the set pH. PAW protein isolates had the highest foaming stability at pH 5, which can be attributed to their enhanced solubility compared to other protein isolates. MSE-PAW particularly showed enhanced foaming stability at pH 7, which was significantly higher than that of any of the other protein isolates, while its control, MSE-C, also showed significantly higher foaming stability at pH 9. Foaming stability ensures consistency and maintains product quality during shelf-life storage. Protein's flexibility in forming a viscoelastic film around air bubbles improves foaming stability. PAW-incorporated extractions maintained foam stability, although it decreased at pH 9. Hou et al. (2025) found that cold plasma application decreased foaming properties by reducing the surface properties of modified protein isolates.

4.4.5.3 Emulsifying Properties

The Emulsifying Activity Index (EAI) of the protein isolates is shown in fig. 19 (c) below. The EAI is a quantitative measure that indicates the ability of the emulsifier to form an emulsion, expressed as the area of the oil-water interface stabilized per unit weight of protein. While EAI values for all protein samples tested remained similar at pH 3, PAW-derived protein isolates showed higher emulsifying activity indices than the other isolates at pH 5 and pH 7, which can be attributed to their increased solubility and surface hydrophobicity. Modified extraction methods

with PAW incorporation did not show distinct differences in the EAI compared to the control at pH 3 and 5, although some increases in the EAI at pH 5 and 7 were observed in MSE-PAW and DES-PAW compared to the control. However, at pH 9, ANOVA indicated significant effects of not only the different extractions employed ($p=0.001$) but also of PAW incorporation ($p=0.000$). ALK PAW ($14.87 \text{ m}^2\text{g}$) and MSE-PAW ($13.06 \text{ m}^2\text{g}$) had significantly higher EAI compared to their controls, ALK C ($11.88 \text{ m}^2\text{g}$) and MSE C ($10.72 \text{ m}^2\text{g}$). This shows the positive impact of including PAW in these methods on the emulsifying properties of the extracted protein isolates. Given the increased surface activity of the modified protein isolates, with improved surface hydrophobicity and oil-holding capacity, the increased emulsifying activity was expected. Sun et al. (2025) observed a similar trend, where the emulsifying capacity of soy protein isolate increased after PAW treatment for 5-30 minutes, after which it decreased. Emulsifying activity is important in several food applications, such as fortified beverages, mayonnaise, and other emulsion-based systems, including nanocomposite applications.

The Emulsifying Stability Index also describes the ability of proteins to resist physical separation mechanisms such as flocculation, creaming, and coalescence. The emulsifying stability index presented in this study indicated that the modified extractions maintained emulsion stability, as they were statistically similar to the control across the pH levels studied. Therefore, an increased emulsifying activity index, along with a similar emulsifying stability index, indicates that incorporation of PAW improved the emulsifying properties of the extracted protein isolates. These results are promising, given that Sun et al. (2025) found that, contrary to the trend in emulsifying capacity, PAW treatment decreased the emulsifying stability of the protein isolates.

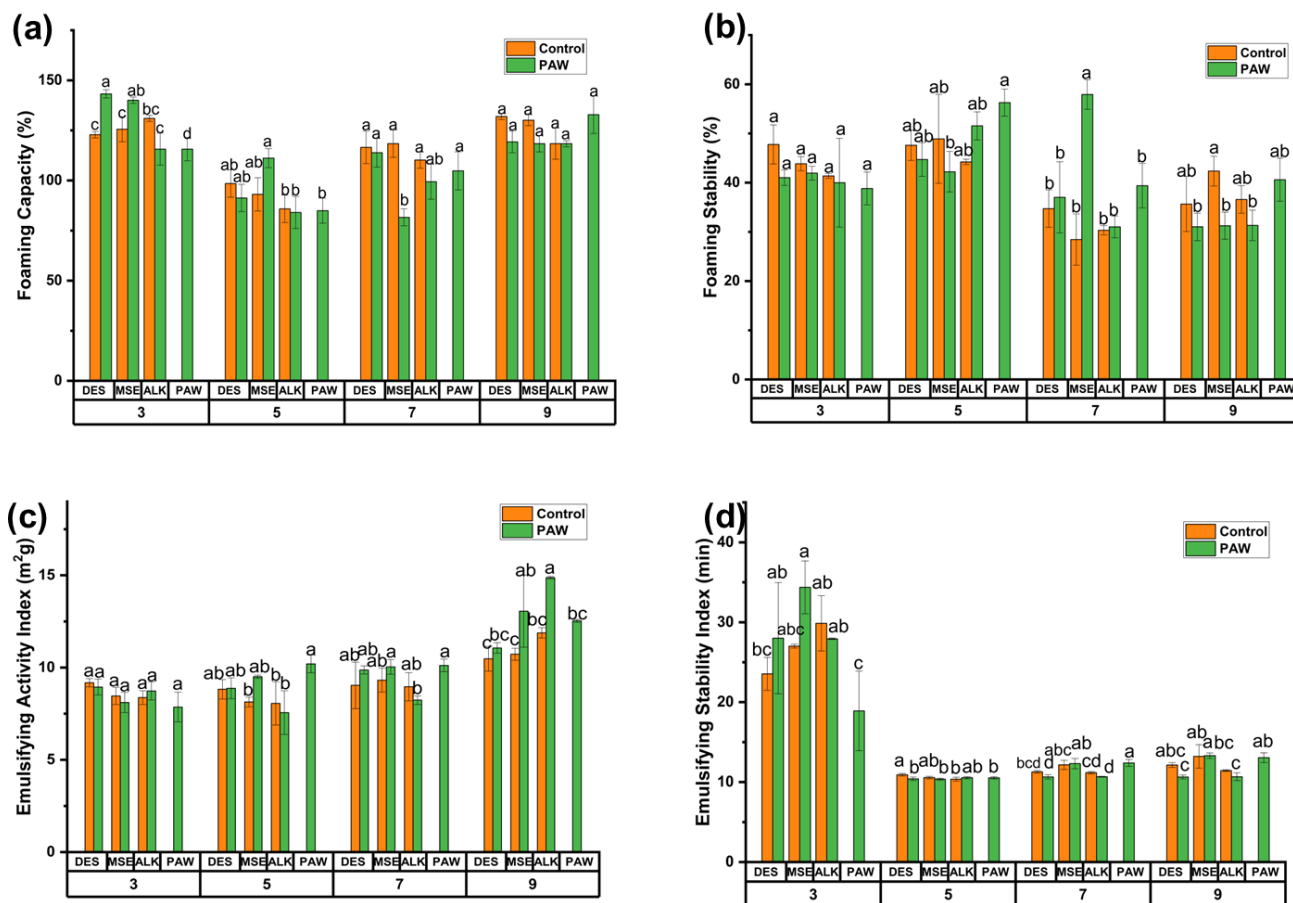


Fig 19: (a) Foaming Capacity (b) Foaming Stability (c) Emulsifying Activity Index (d) Emulsifying Stability Index of Protein Isolates. DES C: Deep eutectic solvent-protein isolates (control), DES PAW: Deep eutectic solvent – protein isolates (PAW), MSE C: Monovalent salt-protein isolates (Control), MSE PAW: Monovalent salt-protein isolates (PAW), ALK C: Alkaline extraction-protein isolates (Control), ALK PAW: Alkaline extraction-protein isolates (PAW), PAW: Plasma Activated Water-Protein Isolates

4.4.5.4. Least Gelling Capacity

The lowest gelling capacity of the chickpea protein isolates is shown in Table 22 below. The least gelling concentration is an inverse measure of gelling capacity, where a higher percentage indicates a poorer ability to form a gel. Changes in pH significantly affected the gelling capacity of the protein isolates studied. While the highest gelling capacity values were observed at pH 3 and 9, pH 5 had the lowest gelling capacity values for all protein samples. This could be due to hindered

disulfide-mediated polymerization at acidic pH, while high pH levels, such as pH 9, owing to the high negative charge on the protein molecules, cause strong electrostatic repulsion that prevents the proteins from aggregating correctly into a cohesive, three-dimensional network. However, aggregation at pH 5 is less controlled due to minimal repulsion, often forming coarse, particulate gels that can be quite stiff but brittle. A similar trend was observed by Tang, Roos & Miao (2024), where the least gelling capacity for whey protein isolate was 12% at pH 3 and 9, while it formed an LGC of 8% at pH 5. PAW-incorporated protein isolates showed varied effects on the LGC across different protein extractions and pH values. MSE-PAW displayed a lower LGC (8%) compared to the control (10%), although ALK-PAW had a much higher LGC (20%) than the control (11%) at pH 3. At pH 5, ALK-PAW formed a gel at 4% while its control had LGC of 6%. These changes require further investigation into the characteristics of the gels formed and how the inclusion of PAW alters the types and strengths of the gels for several applications.

Table 22. Least Gelling Capacity of Protein Isolates

Samples	pH3	pH5	pH7	pH9
DES-C	13%	4%	4%	10%
DES-PAW	14%	4%	5%	10%
MSE-C	10%	3%	4%	6%
MSE-PAW	8%	4%	7%	7%
ALK-C	11%	6%	7%	13%
ALK-PAW	20%	4%	9%	12%
PAW	8%	7%	8%	12%

(Plasma-Activated Water-based extraction), DES-C (Deep Eutectic Solvent – Control), DES-PAW (Deep Eutectic Solvent – PAW infused), MSE-C (Monovalent Salt Extraction – Control), MSE-PAW (Monovalent Salt Extraction – PAW infused), ALK-C (Alkaline Extraction – Control), ALK-PAW (Alkaline Extraction – PAW infused). Data was analyzed using ANOVA and Tukey test and presented in dry basis. Different superscript within a column indicate significant difference

4.4.6. Conclusion

This study systematically investigated the potential of plasma-activated water (PAW) as a sustainable pretreatment for chickpea protein extraction, both as a standalone medium and in combination with established extraction approaches, including alkaline, monovalent salt, and deep eutectic solvent systems. Using a multi-factorial experimental design coupled with a response optimizer, mild plasma conditions (20 kV, 5 minutes) were identified as optimal for generating PAW capable of enhancing protein yield. This finding demonstrates that excessive plasma energy input is unnecessary to achieve effective extraction, thereby highlighting the energy efficiency and practical feasibility of the PAW-assisted extraction approach. PAW incorporated extraction systems maintained extraction efficiency while preserving the molecular integrity and structural hierarchy of chickpea storage proteins, as confirmed from SDS-PAGE. FTIR characterization revealed notable alterations in the protein secondary structure, including increased β -turns and aggregation-prone β -sheet content, suggesting PAW-induced conformational modifications. These structural changes were further supported by SEM micrographs, which showed distinct surface etching, increased roughness, and visible pore formation in PAW-modified protein isolates. Consistently, particle size distribution analysis revealed increased volume- and surface-weighted mean diameters, indicating enhanced protein aggregation. These structural modifications translated directly into functional enhancements, as PAW-treated protein isolates exhibited improved surface hydrophobicity, water and oil holding capacities, foaming capacity and stability, and emulsifying activity index properties that are critical for diverse industrial food applications. Collectively, this work provided mechanistic insight into PAW-protein interactions and established a strong foundation for the development and scale-up of PAW-assisted protein extraction as an energy-efficient and sustainable strategy for next-generation food ingredient production.

4.5. Contribution of Authors

Kofi Oduro: Designing the experiment, Data Analysis and writing – original draft. Dr. Nandika Bandara: conceptualization, supervision, funding acquisition and writing – review and editing

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Chapter 5: Conclusions and future research directions

The development of high-quality chickpea protein isolates via green processing technologies offers a sustainable solution to improve the functionality and value of pulse proteins. Chickpea proteins were successfully extracted and enhanced using Deep Eutectic Solvents (DES) and Plasma Activated Water (PAW), both of which demonstrated significant improvements in protein yield, solubility, and functional attributes. The thesis comprises two studies: the first optimized DES extraction for superior protein recovery and techno-functionality compared to traditional extraction methods, while the second applied PAW pretreatment to further enhance protein yield and functionality. Both methods modified structural integrity, positioning chickpea proteins as viable alternatives for a wide range of food applications.

Summary of findings from the 1st study – Deep Eutectic Solvent – Based Protein Extraction

- The optimized DES condition (liquid: solid ratio of 15:1; Water Content 70%) yielded the highest protein extraction efficiency and reduced solvent use by 11% while increasing the water substitution level by 40% from previous studies (Hewage et al., 2024)
- DES-mediated extraction (53.73%) had a significantly higher extraction yield compared to traditional methods (40.19-45.47%)
- FTIR analysis revealed that DES-CPI had the highest α -helix content (13.86%) and increased low-frequency aggregation-prone β -sheet content (13.36%), while Salt extracted proteins exhibited high-frequency β -sheets unique to their method, indicating favorable structural shifts for enhanced Technofunctionality
- The particle size distribution showed a monomodal distribution for protein isolates, with DES-CPI having the highest D [3,2] (24.7 μ m), indicating a larger surface area than other samples. In contrast, the salt-extracted proteins had the smallest particle size, which correlated with results from SDS-PAGE, where salt-based samples showed bands at lower molecular weight.
- DES-CPI had a uniform morphology in SEM micrographs and enhanced thermal stability with a denaturation temperature of 100.19 ± 1.71 °C
- Proteins from DES showed the highest solubility (82.1%) at pH3, fat holding capacity (199%), and emulsifying activity index (11.98 ± 0.59 m²/g)

- DES-CPI demonstrated significantly higher foaming capacity than other traditional methods from pH 5 to pH 9, while alkaline protein isolates had the lowest at pH 5 and at gelation, with the least gelation concentration consistently at 6% from pH 3 to pH 7.

Summary of findings from 2nd study – Plasma Activated Water (PAW):

- Increasing Voltage (kV) and Time (min) significantly increased the Conductivity (12.22 ± 0.20 – 136.69 ± 10.99 μ S) and the Oxidation Reduction Potential (70.5 ± 7.19 to 188.93 ± 3.27 mV) while the pH reduced (5.57 ± 0.049 to 3.56 ± 0.06)
- The optimal condition for maximizing protein yield (mg/g) was Voltage (20kV) and Time (5min), achieving a composite desirability of 0.79 from the response optimizer.
- While conductivity and oxidation-reduction showed a strong positive correlation (0.805), both also showed strong negative correlations with pH (-0.827, -0.978) and protein yield (-0.308, -0.320), respectively.
- PAW inclusion in the extraction media resulted in no significant changes in the extraction yield, while PAW used alone led to significantly reduced protein purity, yield, and recovery rate
- PAW induced several structural changes, including increased aggregation-prone beta sheets, surface hydrophobicity, and increased particle size distribution
- PAW assisted extraction significantly improved some techno-functional properties, such as the Water Holding Capacity for DES and MSE protein isolates, while both Water and Oil holding capacities were improved in ALK protein samples
- Foaming and Emulsifying properties were significantly improved in the different protein isolates at varying pH levels.

Overall Conclusion:

The findings from both studies underscore the transformative potential of Deep Eutectic Solvents (DES and Plasma Activated Water (PAW) in the sustainable extraction and functional enhancement of chickpea proteins. DES significantly improved the extraction yield, thermal stability, and techno-functional properties, such as solubility, emulsification, foaming, and gelling behavior, which were superior to those of the protein isolates produced via alkaline and salt-based methods.

PAW studies also identified the optimal condition for improved protein yield (mg/g), revealing a correlation between PAW physicochemical properties and protein extraction efficiency, which could be the bedrock for exploiting this technology as a pretreatment to improve protein extraction.

These results establish DES and PAW as effective, eco-friendly technologies capable of addressing the limitations in protein extraction and quality of chickpea proteins. The processed chickpea protein isolates are promising candidates for use in plant-based meat analogues, dairy alternatives, baked goods, and high-protein beverages, contributing to a resilient and sustainable food system.

Future Research Directions:

The current study focuses solely on the structural and functional properties of the extracted protein isolates. Further studies are needed to explore the impact of these technologies on the nutritional properties (digestibility and amino acid profile) of the extracted protein isolates. Pilot studies and assessments of the scale-up feasibility should be conducted to ensure industrial adaptability. The impact of these methods on the toxicological and safety quality of the proteins should be assessed. The impact on antinutritional content and flavor, as well as on microbial quality, especially regarding the incorporation of PAW, should be investigated. Lastly, studies on the reuse and recycling of DES, as well as on the storage stability and chemistry of PAW, are highly encouraged.

Another area worth exploring is the use of these derived ingredients in food products, such as the development of plant-based meat analogues, encapsulation systems, bioactive peptides, and biodegradable packaging materials. This can further highlight the potential of chickpea proteins in these applications.

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Appendix

Appendix 1: Table S3. Analysis of Variance for DES Optimization

Source	DF	Adj SS	Adj MS	F-Value	P-Value
Model	26	156035	6001.4	30.40	0.000
Blocks	2	8287	4143.7	20.99	0.000
Linear	8	144292	18036.6	91.35	0.000
Solid:Liquid Ratio (w/w)	4	143872	35967.9	182.18	0.000
Water Content % (w/w)	4	421	105.2	0.53	0.712
2-Way Interactions	16	3455	216.0	1.09	0.387
Solid:Liquid Ratio (w/w)*Water Content % (w/w)	16	3455	216.0	1.09	0.387
Error	48	9477	197.4		
Total	74	165512			

Appendix 2: Table S3.1. Relative content of secondary structures in extracted chickpea protein isolates

Secondary Structure components	DES-PI		MSE-PI		ALK-PI	
	Peak Center Value (cm ⁻¹)	Relative Content (%)	Peak Center Value (cm ⁻¹)	Relative Content (%)	Peak Center Value (cm ⁻¹)	Relative Content (%)
β-sheets	1619, 1627, 1634	56.55±0.02 ^b	1619,1626, 1633,1693	62.06±0.18 ^a	1619,1626, 1634	55.46±1.89 ^b
Intramolecular β-sheets	16,271,634	46.43±0.02 ^a	1626, 1633	40.44±0.31 ^b		42.13±1.38 ^b
Aggregation prone β-sheet (low freq)	1619	10.12±0.000 ^c	1619	11.45±0.15 ^b	1619	13.34±0.51 ^a
aggregation prone β-sheet (high freq)	-	-	1693	10.12±0.002	-	-
Unordered Structures	1644	5.79±0.53 ^a	1644	6.42±0.001 ^a	1645	5.85±0.61 ^a
α-helix	1650	13.30±1.51 ^a	1650	8.75±0.08 ^b	1650	14.15±1.74 ^a
Unordered Helix	1662	14.38±1.20 ^a	1665	14.14±0.07 ^a	1667	12.99±0.30 ^a
β-turns	1681	9.98±0.23 ^b	1682	8.68±0.02 ^b	1681	11.55±1.05 ^a

All data were analyzed using ANOVA and Tukey test. Different superscripts within the column indicate a significant difference ($P<0.05$).

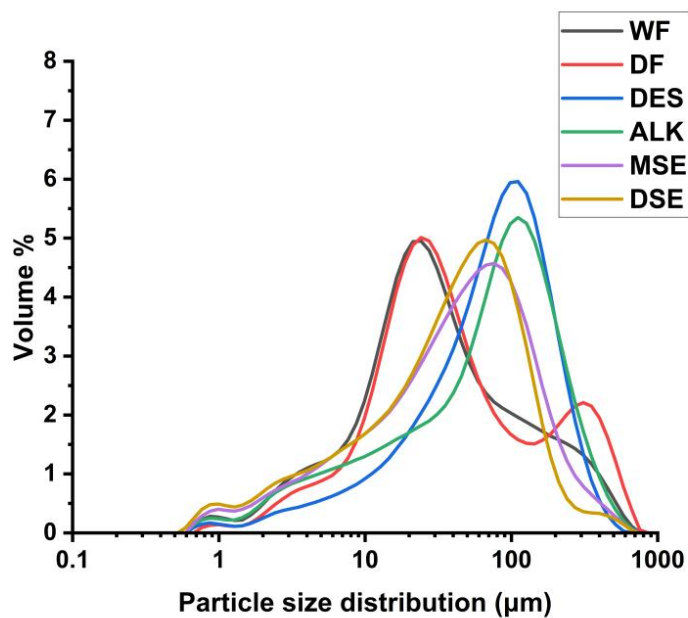
DES-PI=Deep eutectic solvent-extracted protein isolates, MSE-PI = Monovalent salt extracted protein isolates; ALK-PI=Alkaline extracted protein isolates.

Appendix 3: Table S3.2. Relative content of secondary structures in extracted chickpea protein isolates

Secondary Structure	DF		WF	
	Peak Center Value (cm^{-1})	Relative content (%)	Peak Center Value (cm^{-1})	Relative content (%)
β -sheet	1627,1629, 1638	49.18 $\pm$ 5.22 ^a	1619, 1626, 1633,1693	45.56 $\pm$ 3.12 ^a
Intramolecular β -sheets	1627, 1629, 1638	49.18 $\pm$ 5.22 ^a	1626, 1633	45.56 $\pm$ 3.12 ^a
aggregation prone β -sheets (low freq)	-	-	-	-
aggregation prone β -sheet (high freq)	-	-	-	-
Unordered Structures	-	-	-	-
α -helix	1650	15.16 $\pm$ 1.98 ^b	1650	25.69 $\pm$ 3.85 ^a
Unordered Helix	1660,1668	24.06 $\pm$ 2.51 ^a	1662	16.45 $\pm$ 1.24 ^b
β -turn	1681	11.60 $\pm$ 0.90 ^a	1682	12.31 $\pm$ 0.07 ^a

All data were analyzed using ANOVA and Tukey test. Different superscripts within the column indicate a significant difference ($P<0.05$). DF= defatted flour; WF=Whole Flour

Appendix 4: Fig S3.3. Particle size distribution of chickpea samples



Appendix 5: Table S4. Analysis of Variance for Conductivity

Source	DF	Adj SS	Adj MS	F-Value	P-Value
Model	17	55235.9	3249.17	68.37	0.000
Blocks	2	208.9	104.45	2.20	0.129
Linear	6	44214.5	7369.08	155.06	0.000
Voltage	3	22149.9	7383.31	155.36	0.000
Time	3	22064.5	7354.85	154.76	0.000
2-Way Interactions	9	10812.5	1201.39	25.28	0.000
Voltage*Time	9	10812.5	1201.39	25.28	0.000
Error	30	1425.7	47.52		
Total	47	56661.6			

Appendix 5: Table S4.1. Analysis of Variance for Oxidation-Reduction Potential

Source	DF	Adj SS	Adj MS	F-Value	P-Value
Model	17	72392	4258.3	21.95	0.000
Blocks	2	1002	500.8	2.58	0.092
Linear	6	67939	11323.2	58.37	0.000
Voltage	3	33104	11034.5	56.89	0.000
Time	3	34836	11611.9	59.86	0.000
2-Way Interactions	9	3451	383.4	1.98	0.078
Voltage*Time	9	3451	383.4	1.98	0.078
Error	30	5819	194.0		
Total	47	78211			

Appendix 6: Table S4.2. Analysis of Variance for pH of PAW

Source	DF	Adj SS	Adj MS	F-Value	P-Value
Model	17	22.5669	1.32746	43.30	0.000
Blocks	2	0.2056	0.10281	3.35	0.048
Linear	6	21.7206	3.62009	118.07	0.000
Voltage	3	10.4250	3.47499	113.34	0.000
Time	3	11.2956	3.76520	122.81	0.000
2-Way Interactions	9	0.6407	0.07119	2.32	0.040
Voltage*Time	9	0.6407	0.07119	2.32	0.040
Error	30	0.9198	0.03066		
Total	47	23.4866			

Appendix 7: Table S4.3. Relative content of secondary structures in extracted chickpea protein

Secondary Structure components	Peak Center Value (cm-1)	DES-C	DES-PAW	MSE-C	MSE-PAW
		Relative	Relative	Relative	Relative
		Content	Content	Content	Content
		(%)	(%)	(%)	(%)
β-sheets	1615-1638				
	1690-1700	59.44±0.14 ^a	60.20±0.53 ^a	59.86±0.47 ^a	60.79±1.03 ^a
	Intramolecular β-sheets				
	1625-1638	36.66±0.65 ^{bc}	32.79±1.67 ^c	41.67±0.56 ^{bc}	41.79±1.95 ^{bc}
	Aggregation prone β-sheet (low freq)				
	1615-1625	13.21±0.48 ^{ab}	16.82±0.76 ^a	7.71±0.30 ^c	8.07±0.76 ^{bc}
	aggregation prone β-sheet (high freq)				
	1690-1700	9.58±0.04 ^c	10.00±0.19 ^d	10.48±0.08 ^c	10.94±0.17 ^b
Unordered Structures	1639-1648	5.78±0.60 ^a	6.48±0.61 ^a	5.38±0.40 ^a	5.23±1.25 ^a
α-helix	1649-1660	12.51±0.29 ^b	11.13±0.30 ^b	12.10±0.21 ^b	11.07±0.24 ^b

Unordered

1660-1679

13.02 ± 0.11^c

13.02 ± 0.11^c

13.64 ± 0.07^b

13.95 ± 0.27^b

Helix

β -turns

1680-1690

9.55 ± 0.06^b

9.18 ± 0.10^{cd}

9.02 ± 0.08^d

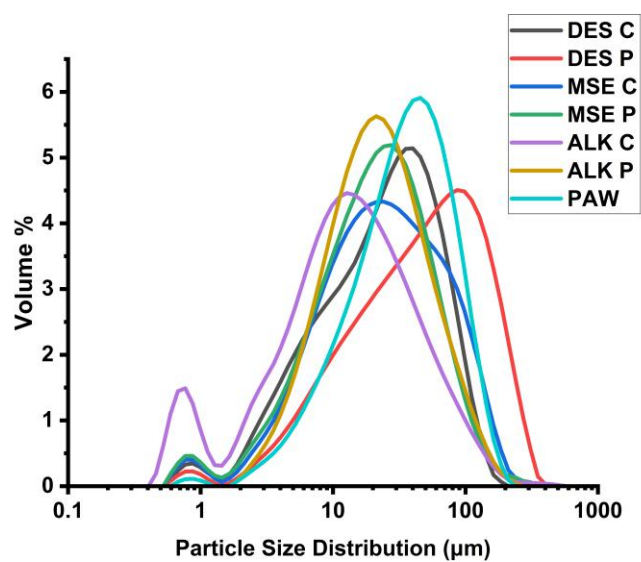
8.97 ± 0.05^d

Appendix 8: Table S4.4. Relative content of secondary structures in extracted chickpea protein

Secondary Structure components	Peak Center Value (cm-1)	ALK-C	ALK-PAW	PAW
		Relative	Relative	Relative
		Content	Content	Content
		(%)	(%)	(%)
β-sheets	1615-1638	63.55±3.39 ^a	60.96±2.35 ^a	59.42±3.41 ^a
	1690-1700			
	Intramolecular β-sheets	1625-1638	46.37±6.48 ^{ab}	43.67±6.35 ^b
	Aggregation prone β-sheet (low freq)	1615-1625	5.71±3.17 ^c	5.57±3.81 ^c
	aggregation prone β-sheet (high freq)	1690-1700	11.47±0.112 ^a	11.73±0.21 ^a
Unordered Structures	1639-1648	4.87±0.46 ^a	4.69±1.67 ^a	-
α-helix	1649-1660	10.74±0.23 ^b	11.81±1.67 ^b	20.24±1.57 ^a

Unordered				
Helix	1660-1679	14.62±0.04 ^a	14.75±0.41 ^a	10.25±0.01 ^d
β-turns	1680-1690	9.29±0.09 ^c	9.36±0.03 ^{bc}	10.09±0.11 ^a

Appendix 9: Table S4.5. Particle Size distribution of chickpea protein isolates



Particle Size Distribution of Protein Isolates. DES C: Deep eutectic solvent-protein isolates (control), DES PAW: Deep eutectic solvent–protein isolates (PAW), MSE C: Monovalent salt-protein isolates (Control), MSE PAW: Monovalent salt-protein isolates (PAW), ALK

C: Alkaline extraction-protein isolates (Control), ALK PAW: Alkaline extraction-protein isolates (PAW), PAW: Plasma Activated Water-Protein Isolates