

Optimizing Sustainable Physical Processing Technologies for Functionality Improvement in Pulse Proteins

by

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Abstract

The increasing demand for sustainable, plant-based protein ingredients has driven the development of environmentally friendly processing technologies that enhance protein concentration and functionality while minimizing resource use. This thesis investigates the combined application of dry fractionation and non-thermal processing approaches to improve the quality and functionality of plant protein concentrates. In the first study, air classification was employed as a solvent-free, dry fractionation technique to produce protein-enriched fractions from green lentil flour while preserving the native protein structure. The effects of dehulling (dehulled/ whole seeds) and air classification wheel speed (30/40 Hz) on Proximate composition, particle size, amino acid profile, secondary structure, thermal behavior, functional properties, antinutritional factors, and in vitro protein digestibility were systematically evaluated. The protein and crude fat contents of the concentrates increased at 40 Hz and were within the ranges of $51.18 \pm 0.44\%$ - $54.29 \pm 0.45\%$ and $2.03 \pm 0.01\%$ - $2.35 \pm 0.06\%$, respectively. The lowest particle size ($D_v(10)=2.45 \mu\text{m}$, $D_v(50)=6.47 \mu\text{m}$, $D_v(90)=14.3 \mu\text{m}$) was recorded by the DH samples. Significant effects ($p < 0.05$) of the two variables on techno-functional properties, thermal behavior, amino acid composition, and digestibility (IVPD) were observed. In terms of techno-functional properties; water-holding capacity (WHC), oil-holding capacity (OHC), protein solubility (PS), emulsion capacity and stability (EC and ES), Foaming capacity and stability (FC and FS), it was observed that. dehulling increased PS at pH 5 and ES at pH 3, decreasing OHC, PS at pH 7, EC at pH 3 and 9, and ES at pH 5. Higher speed increased PS at pH 3 and 5, EC at pH 3 and reduced PS at pH 7 and 9, ES at pH 5. FC was higher at pH 3 and 5, while FS was better at pH 7. Dehulling improved the IVPD by 1.6-2.5%. These observations underscore , the importance of determining their optimal combinations for specific applications. The second study was allocated to optimizing and assessing

the potential of dielectric barrier discharge cold plasma treatment (DBDCP) as a form of High Voltage Atmospheric Cold Plasma (HVACP) treatment to further enhance the properties of the concentrates. It was optimized based on treatment time (1/2/5/10/15 min) and voltage (15/20 kV), with carbonyl formation and protein solubility as response variables. DBDCP treatment positively influenced techno-functional properties as follows. Protein solubility was enhanced at pH 3, while oil-holding capacity increased approximately 2-fold upon treatment. Emulsifying and foaming properties were unaffected except for a decline in stability at pH 3. DBDCP treatment did not affect water holding capacity. Secondary structure analysis showed a predominance of β -sheet content (46.36 ± 0.01 - $53.14 \pm 0.61\%$) with an increasing trend as the treatment increased. Aggregates (8.521 ± 0.19 - $10.626 \pm 0.63\%$) were observed only in treated samples, indicating denaturation caused by DBDCP treatment. DBDCP treatment at 1min/15kV significantly enhanced the contents of arginine, glycine, and tyrosine, while SDS-PAGE analysis showed no variation in the molecular weight distribution. Morphological evaluation revealed particle fragmentation, increased surface roughness, and surface etching at higher time–voltage combinations. Furthermore, the dual function of DBDCP in improving functionality and food safety was established through achieving a 2 log CFU/g reduction per cycle at 20 kV for *E. coli*. . The results demonstrate that the combined use of air classification and cold plasma treatment highlights the potential of integrating sustainable dry fractionation with non-thermal plasma technology to develop high-quality plant protein ingredients with enhanced functionality. Overall, this work provides valuable insights into green processing strategies for next-generation protein ingredients suitable for food and industrial applications.

Keywords: Sustainability, Protein concentrates, Lentils, Dry fractionation, High Voltage
Atmospheric Cold Plasma Technology

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Dedication

To my dearest Ammi,

For bestowing upon me the gifts of dedication, endurance, and compassion,

To my precious Babiini,

For showing me how beautiful it feels to find strength in love,

and to all those who were part of this adventurous journey,

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List of Abbreviations

AAS – Amino Acid Score

DBD – Dielectric Barrier Discharge

DF – Dry Fractionation

DH – Dehulled

DSC – Differential Scanning Calorimetry

EAI – Emulsion Activity Index

ES – Emulsion Stability

FC – Foaming Capacity

FS – Foaming Stability

FTIR – Fourier-Transformed Infrared Spectroscopy

HVACP – High Voltage Atmospheric Cold Plasma

IVPD – In Vitro Protein Digestibility

NDH – Non-dehulled

OHC – Oil Holding Capacity

PC – Protein Concentrate

PDCAAS – Protein Digestibility Corrected Amino Acid Score

PI – Protein Isolate

SDS-PAGE - Sodium Dodecyl Sulfate–Polyacrylamide Gel Electrophoresis

WE – Wet Extraction

WHC – Water Holding Capacity

Chapter 1: Introduction

1.1 Research Background

The changing food industry landscape demands the incorporation of novel food items into diets and novel food ingredients into food products. Therefore, plant protein ingredients are becoming essential ingredients in protein-based foods. Despite challenges with their functionality, these ingredients are rapidly being adopted in the food industry.

Most of these plant protein ingredients, namely concentrates and isolates, are effectively incorporated into plant-based meat alternatives, thereby meeting the protein requirements of these products. These ingredients are increasingly recognized as valuable vegan sources of amino acids, aligning well with the growing popularity of vegan, vegetarian, and flexitarian dietary trends (Prasad et al., 2025). This is also driven by other factors, such as environmental concerns about greenhouse gas emissions, extensive land and water use, and inefficient energy use. It has been calculated that a dietary shift towards a vegan diet can reduce GHG emissions by 4-20%, whereas a transition to a vegetarian diet can reduce them by 12% (Hallström et al., 2015). Several health concerns raised over the nutritional quality and safety of animal meat, such as process-induced toxins, saturated fatty acids, heme iron, and meat-borne infections, have also contributed to the trends favoring plant-based meat consumption (He et al., 2020). At the same time, animal welfare organizations encourage consumers to consider plant-based alternatives to meat products due to concerns about animal welfare throughout production, transport, and slaughter processes (Santo et al., 2020). Apart from meat analogs, numerous applications of plant protein concentrates effectively leverage the favorable nutritional and techno-functional properties of plant proteins. Pulse proteins are being used in a wide array of applications, including nutritional supplements, beverages, bakery and confectionery, as well as animal feed (Fatima et al., 2024). It is evident that

plant protein concentrates are gaining increased attention in the current food industry, and it is important to identify plant protein sources with higher nutritional and functional properties while experimentally evaluating the most suitable modification methods to enhance their properties.

Protein concentrates are mainly produced through dry fractionation, which frequently involves milling and air classification. The density and size of particles determine their aerodynamic behavior, which in turn determines the air classification efficiency of the process. Upon air classification, the material is separated into fine and coarse fractions based on the cut size set for the process. Cut size is the specific particle size threshold at which a particle has an exactly equal (50%) chance of ending up in either the fine fraction or the coarse fraction. Particles such as starch granules with a size above the cut size move into the coarse fraction, while smaller particles such as protein particles move into the fine fraction (Schutyser & van der Goot, 2011). Apart from the size, the powder mixture's dispersibility also impacts the air classification efficiency. Dry fractionation can be improved through several processes, such as milling the finer fraction, adjusting air classifier wheel parameters, applying plant breeding techniques to alter grain characteristics, and pretreating grains (Pelgrom et al., 2015).

Several technological interventions have proven effective in improving the functionality of protein concentrates, thereby enhancing their performance and applications in food systems. They can be classified as physical, chemical, and biological techniques, among which nonthermal technologies are gaining increasing attention. Cold plasma technology is a promising approach that can modify proteins, rendering them more functional, increasing digestibility, and reducing allergenicity. Moreover, this technology falls within the sustainable category and is considered a green processing technology (Olatunde et al., 2023). This technology is based on the conversion of carrier gases into the fourth state of matter, plasma, while producing various species, including

negative and positive ions, radicals, neutral and excited molecules, atoms, electrons, and photons, by providing sufficient energy (Bahrami et al., 2022). The most noteworthy advantage of this method is that it modifies the protein without causing thermal damage.

Numerous plant protein sources have been identified as suitable for producing plant protein concentrates. Among these, lentils are an essential source for the Canadian food industry, as Canada is the world's largest producer and exporter of lentils (Aghababaei et al., 2024), with its favorable Leucine/Isoleucine and Leucine/Lysine ratios and high digestibility (Jarpa-Parra, 2018). At the same time, it bears significance as a sustainable plant protein source with a rich amino acid profile (Domínguez et al., 2023). However, poor techno-functional properties hinder their optimal use as protein concentrates in food applications. Thus, researching the effects of HVCAP on lupin and lentil protein concentrates is justified by its potential to overcome current functionality and digestibility related limitations, offer novel processing solutions, meet industry needs for improved plant proteins, and contribute to more sustainable and economically viable protein production.

1.2 Hypothesis

This research hypothesizes that optimizing optimizing dry fractionation processing parameters (dehulling as a pretreatment and air classifier wheel speed) and nonthermal modifications (High Voltage Atmospheric Cold Plasma) can improve the functional properties of pulse protein concentrates.

1.3 Objectives

Study 1 - Optimizing the production process of lentil protein concentrates for functionality improvement

- To optimize dry fractionation conditions for dry fractionated lentil protein concentrates

- To evaluate the impact of dry fractionation on the chemical composition, structure, and techno-functionality of the protein concentrates

Study 2 – Optimizing the nonthermal processing of dry fractionated protein concentrates using HVACP

- To optimize HVACP processing technology for dry fractionated lentil protein concentrates
- To evaluate the impact of HVACP on techno-functional properties, surface properties, and digestibility of the protein concentrates
- To explore the dual functionality of HVACP in improving the microbial safety of protein concentrates

Chapter 2: Literature Review

2.1 Overview of the Plant Protein Industry

2.1.1 Global Protein Consumption Landscape

Dietary patterns vary significantly across geographical regions and communities and continue to evolve for various reasons. Protein consumption, in particular, changes frequently as advances in technology, sustainability principles, and nutrition science are integrated into food systems. The main sources of dietary protein are animal- and plant-based proteins, with fungal- and bacterial-based proteins also contributing. However, the contribution of each food type to overall protein intake varies across global regions (Smith et al., 2024). Animal-based proteins have become more prominent in many regions, particularly in developed countries. Meats that contribute to animal-based protein intake are mainly categorized as red meat (bovine, mutton, goat, etc.) and white meat (pork and poultry), and their consumption is projected to rise in the coming years. Trends also show that consumption is shifting towards poultry, as reflected in lower prices in developing countries (Henchion et al., 2021).

In the United States, meat consumption accounts for most of daily protein intake (Fehrenbach et al., 2016). Regarding per capita availability, chicken has shown a steady upward trend since 1940, surpassing pork in 1996 and beef in 2010, and has thus become the most available animal-based protein source for US citizens (USDA, 2024). Based on data from the 2015 Canadian Community Health Survey – Nutrition, the average protein intake among Canadians was 79.47 ± 0.70 g/d and 1.20 ± 0.01 g/kg of BW/d. This accounted for $16.96 \pm 0.11\%$ of the total energy intake. Animal protein sources, red meat and processed meats, poultry, and eggs were the major protein sources, which were followed by cereals, grains, and bread (Auclair & Burgos, 2021). In the European Union, animal proteins are the main source of protein in food, whereas plant proteins are the main

source in animal feed (Albaladejo, 2023). Plant proteins make up a significant portion of daily protein intake across Africa and Asia. According to the FAO Food Balance Sheets 2020, Western, Eastern, and Central Africa have the highest proportions of plant-based proteins in their daily diets, at 76%, 75%, and 70%, respectively. In the Asian region, Southern Asia leads, with plant-based proteins accounting for 74% of daily protein intake (Vissamsetti et al., 2024). Therefore, it is evident that protein consumption patterns vary widely across regions. At the same time, these differences can be correlated with the incidence of protein-related malnutrition, especially within developing countries.

However, the consumption of plant-based protein foods is increasing, driven by various factors. Mainly, rising meat prices have made meat products inaccessible to populations in lower-income developing countries, prompting a shift toward plant-based protein sources. The price of beef has increased by 2.4 times over the past 20 years, according to data published by the World Bank (Dolganyuk et al., 2023). Increasing prices have become the leading factor in declines in red meat consumption in countries like Uruguay (Realini et al., 2022), Mexico (Estévez-Moreno et al., 2021), and (Hase et al., 2023). Price is an important driver of meat consumption in many countries, including China, France, Brazil, Cameroon, South Africa (J. Liu et al., 2023), and the United States (Tonsor & Lusk, 2022). Environmental impact of animal-based diets is another factor stimulating the changes in the consumption patterns. The negative environmental impacts of these diets can be multifaceted and include greenhouse gas (GHG) emissions, land use and degradation, scarcity-weighted water use, nutrient pollution, deforestation, and more (Espinosa-Marrón et al., 2022). Among meat types, ruminant meat produces the most emissions, followed by non-ruminant meat and poultry. It has also been reported that adopting a flexitarian diet significantly reduces one's carbon footprint and thus complements technology use and management strategies to reduce

carbon footprint in food systems (Ferrari et al., 2022). Meat production is responsible for the emission of three main anthropogenic greenhouse gases: carbon dioxide (CO₂), methane, and nitrous oxide (N₂O). Though livestock grazing has several benefits such as enhancement of soil C sequestration (Henderson et al., 2015) and increased N availability for plant growth due to livestock excreta (Gerber et al., 2013) it is essential to balance these effects with many negative impacts such as direct GHG emissions of livestock, erosion, deforestation, and loss of natural plant covering (Godfray et al., 2018). The resource requirements for meat production have been quantified as higher than those for plant proteins, underscoring the meat industry's lack of sustainability. Compared with soybean protein production, meat has a land requirement that is 6-17 times greater, a water requirement that is 26 times greater, and a fossil fuel requirement that is 6-20 times greater. Similar trends are also evident when comparing cow milk-based cheese and lupin-based cheese (Reijnders & Soret, 2003). From an environmental impact perspective, consumer attitudes and beliefs also influence the consumption of animal-based foods. Religion, cultural values, and social ethics significantly influence this regard. In a study comparing the perspectives of US citizens and Europeans on plant-based diets, it was noted that Seventh-day Adventists in the US tended to choose their diets based on religious beliefs, especially lacto-ovo-vegetarian and vegan diets (Fresán et al., 2020). Similar trends were observed among university students in the southwestern United States, indicating that they are more motivated to adopt plant-based diets when these diets align with their ethical beliefs (Vizcaino et al., 2021). In addition, it has been found that stimulating regret or disgust toward animal-based food products has negatively affected meat consumption. Disgust-oriented messages were more effective than health-oriented messages in persuading consumers to avoid meat consumption (Palomo-Vélez et al., 2018). Consumers' perceptions of animal farms also affect sensory properties and the overall eating

experience, indicating that meat consumption is reduced when consumers believe the animals were raised in conditions such as confinement in indoor pens (Anderson & Barrett, 2016). Thus, many factors are driving a trend toward plant-based food consumption.

2.1.2 Plant Protein Ingredients

Currently, the two main plant protein ingredients are protein isolates and concentrates. They differ in their protein contents. It is generally accepted that protein concentrates contain 65-90% protein, whereas protein isolates contain more than 90% protein (Codex, 2022; Meganaharshini et al., 2023). Protein isolates offer a unique advantage due to their high protein content and are frequently incorporated into food formulations, including infant food, beverages, and texturized protein products. Protein concentrates are utilized in dairy products, soups, sauces, and nutritional supplements. Plant protein concentrate and isolate production occur via a process that integrates multiple technologies for milling, protein extraction, drying, and preservation (Fatima et al., 2024).

Protein extraction techniques are mainly of two types: dry and wet extraction. Wet extraction methods are commonly used to extract proteins. They involve several steps, including preparing the flour suspension, extracting it under acidic or alkaline conditions, performing isoelectric precipitation or ultrafiltration, and drying (Rivera et al., 2024). They offer the benefit of producing protein isolates with relatively high purity, but they have raised many environmental concerns due to extensive water, energy, and chemical solvent use. Dry extraction relies on air classification, which uses particles' aerodynamic properties (Schutyser & van der Goot, 2011). Owing to the growing environmental awareness, dry fractionation is gaining attention as a sustainable alternative to wet extraction methods. However, information on dry fractionation procedures and their optimization remains lacking in the literature (Pulivarthi et al., 2023). However, it is important to investigate ways to optimize the dry extraction process and modify protein

concentrates, as these measures can significantly contribute to the sustainability of the protein industry.

2.2 Lentils as a Protein Source

2.2.1 Growth and Production Landscape of Lentils

Lentils (*Lens culinaris*) are among the oldest cultivated legumes, have been incorporated into cuisines for a long time, and have been an integral part of the diets of people in more than 70 countries worldwide (Nosworthy et al., 2017). Lentils can be yellow, red, or green, whereas the market is mostly dominated by red and green lentils, which are consumed as split or whole seeds. The annual production rate of lentils has kept a higher pace over the past decades, surpassing that of peas, chickpeas, and common beans, indicating increasing demand and consistent consumption worldwide. This is mainly attributed to the shorter cooking time compared with the pulses (Khazaei et al., 2019). Lentils also have agronomic significance due to their resistance to drought, their ability to be cultivated in a wide range of soils, and their nitrogen-fixing ability (Montejano-Ramírez & Valencia-Cantero, 2024). It also serves as a highly suitable crop for crop diversification, further encouraging its growth and production worldwide (Gan et al., 2017). At the same time, lentils have been successfully incorporated into intercropping systems with crops such as mustard (Devi et al., 2014), linseed (Sarkar et al., 2001), and sugarcane (Rana et al., 2001). Lentil cultivation has also been identified and practiced as a livelihood strategy for populations in arid regions, while simultaneously improving soil conditions (Thornton & Cramer, 2012).

Canada ranks as the leading producer of lentils, accounting for approximately 44% of global production, followed by India with 18% (Aghababaei et al., 2024). According to statistics published by the Food and Agriculture Organization, lentil cultivation improved significantly

during 2010 – 2021, as indicated by production quantities and the land area dedicated to lentil production (Aghababaei et al., 2024). Myriad economic and social factors, such as affordability, availability, and lower environmental impact during cultivation, could drive this. (Montejano-Ramírez & Valencia-Cantero, 2024). The growing demand for sustainable plant-based protein sources has also favored lentil production and expanded its use in various food applications (Brishti et al., 2023). As concerns grow over the environmental impact of animal-based proteins, lentils present an attractive alternative due to their agronomic significance, while encouraging their value addition and enhanced production.

2.2.2 Nutritional Composition of Lentils

Lentils are rich sources of proteins, as they contain approximately 20.6-31.4% proteins (Khazaei et al., 2019). Lentil proteins are composed of four main fractions as 70% globulins (7S and 11S), 16% albumins, 11% glutelins, and 3% prolamins (Joehnke et al., 2021). Lentil proteins generally exhibit relatively low average molecular weights (MWs), with glutelins ranging from 17–46 kDa, albumins around 20 kDa, and prolamins between 16–64 kDa. The globulin fraction can be further classified into two main components: legumin and vicilin. Legumin has a higher molecular weight, typically 320-380 kDa, and is composed of six polypeptide subunits. Each subunit consists of an approximately 40 kDa acidic chain and a 20 kDa basic chain, which are linked together through disulfide bonds (Aghababaei et al., 2024). In contrast, vicilin (the 7S globulin fraction) has a molecular weight of approximately 50–60 kDa and consists of glycosylated subunits that contain about 2.8% carbohydrate (Alrosan et al., 2022).

Lentil proteins provide sufficient amounts of most essential amino acids; however, they are relatively deficient in cysteine, methionine, and tryptophan, making these the limiting amino acids (Khazaei et al., 2019). The relative abundance of amino acids in lentils may vary by variety, yet

these amino acids remain limiting (Kahraman, 2016). This limitation can be addressed by combining lentil proteins with cereal proteins, thereby achieving a more balanced and complete amino acid profile (Abdel-Aal, 2023).

In addition, lentils are legumes characterized by higher fiber and lower fat content. Brummer et al. (2015) recorded relatively higher total soluble fiber in lentils, when compared to peas and chickpeas. The total dietary fiber content of green lentils amounts to approximately 11%, which enhances the nutritional value of lentils (Dhull et al., 2023). Studies by Zare et al. (2012) have outlined the beneficial effects of lentils' high dietary fiber content in regulating bowel movements and promoting a healthy gut microbiota. In addition, the reduced acute blood glucose and insulin response to lentil consumption has also been attributed to lentils' higher dietary fiber content (Clarke et al., 2022).

Lentils also remain significant due to their relatively higher levels of bioactive phytochemicals, including flavonoids, phenolics, carotenoids, tocopherols, saponins, phytic acid, and phytosterols (Dhull et al., 2023). Therefore, lentils are often associated with numerous health benefits against various degenerative diseases, and many studies are being conducted to confirm their potential as a functional food (Mustafa et al., 2022). It has also been found that these compounds are mostly concentrated in lentil hulls, especially in phenolics, flavonoids, and condensed tannins (Guo et al., 2023). However, these compounds are largely affected by various processing methods, such as cooking, germination, fermentation, dehulling, milling, and extrusion, which can also be beneficial by reducing the levels of antinutritional phytochemicals that obstruct the digestibility and bioavailability of proteins (Dewan et al., 2024).

2.2.3 Extraction and Processing of Lentil Proteins

Despite being frequently consumed as whole or split seeds, lentils are now being explored as protein ingredients in a wide range of food products. This trend has drawn significant attention to various protein extraction methods, particularly dry and wet extraction techniques, to produce lentil protein ingredients for use in meat analogs, gluten-free products, and other applications (Siddiq et al., 2022).

2.2.3.1 Dry Fractionation of Lentil Proteins

Dry fractionation is emerging as a promising protein extraction method and is being extensively explored due to several concerns associated with conventional wet extraction methods. These include heavy chemical and waste generation, negative environmental impact, and increased levels of denaturation induced by the harsh conditions used in the extraction process (Schutyser & van der Goot, 2011). Among dry fractionation techniques, the most frequently used method is air classification, which involves milling and subjecting the flour to an air stream to facilitate the separation of larger starch granules from smaller protein particles based on their size and density (Schutyser et al., 2015). This technique is especially suitable for pulse protein sources such as lentils, as they exhibit distinct differences in the sizes of starch and protein particles, facilitating efficient separation via air classification (Funke, Boom, et al., 2022).

Funke, Loeffler, et al. (2022) assessed the air classification of lentils with special emphasis on the effect of the air classifier wheel speed, and observed that the increasing wheel speed can improve the protein enrichment in the protein concentrates obtained. They achieved protein contents of 65.8% and 55.3% by operating the air classifier at 11,000 and 9,000 rpm wheel speeds, respectively. These values are higher than other values reported in the literature. . It was attributed

to the sample's higher initial protein content. Pulivarthi et al. (2025) obtained protein concentrates with 54–60% protein by increasing the protein content at higher wheel speeds, using red lentils as the protein source. They also reported that the abundance of hydrophobic amino acids in dry-extracted protein concentrates remained lower than in wet-extracted protein isolates, thereby contributing to better functional properties in the concentrates. Parallel to protein enrichment, air classification can also result in the enhancement of other micronutrients, such as minerals, namely P, S, K, Mn, Fe, Ni, and Cu, as well as antinutritional factors, such as total phytates and stachyose, in different lentil varieties (De Angelis et al., 2024). Furthermore, protein enrichment during dry fractionation was further enhanced by adding a subsequent triboelectric separation step, resulting in a 6% higher enrichment of lentil proteins (Xing et al., 2020). Accordingly, dry fractionation can effectively produce protein ingredients with acceptable purity while minimizing the environmental impact and preserving native protein functionality. However, the protein content achieved through dry fractionation is typically lower than that obtained through wet processing, posing a challenge for producing ingredients suitable for exclusively high-protein food products.

2.2.3.2 Wet extraction of lentil proteins

Wet extraction methods are more commonly used to produce pulse protein ingredients, including lentil proteins. There are conventional wet extraction methods, such as alkaline extraction and salt-induced extraction, while novel methods are also being extensively studied at present (Aghababaei et al., 2024). Alkaline extraction of proteins employs the fact that protein solubility generally increases under strongly alkaline conditions, as protein molecules carry a higher negative charge, leading to increased electrostatic repulsion between them and improved dispersion in solution. In contrast, near the isoelectric point, protein solubility decreases because the net charge on protein molecules is minimal, leading to weak electrostatic repulsion and increased aggregation (Shrestha

et al., 2021). Accordingly, protein is initially solubilized at alkaline pH and allowed to precipitate by lowering the pH to approximately 4.2, thereby facilitating precipitation at the isoelectric point (Lee et al., 2007). By optimizing the alkaline extraction method for lentil proteins, Jarpa-Parra et al. (2014) achieved protein ingredients with 82% protein, significantly higher than those of dry-extracted protein concentrates. Similarly, Karaca et al. (2011) Karaca et al. (2011) also obtained protein ingredients with 82% protein content by subjecting lentil flour to isoelectric precipitation following alkaline solubilization.

Another frequently utilized wet-extraction method is salt-assisted extraction. Certain plant proteins, particularly globulins, can be solubilized in salt solutions such as sodium, calcium, or potassium chloride at near-neutral pH. These proteins can subsequently be recovered either by reducing the ionic strength through dilution, which induces precipitation, or by employing membrane separation techniques (Aghababaei et al., 2024). Karaca et al. (2011) observed that the salt extraction was less efficient for protein extraction than isoelectric precipitation, as the protein content of the salt-extracted ingredient remained at 74%. Accordingly, the extraction method plays a significant role in determining the protein composition of the ingredient, thereby influencing its techno-functional properties and its applicability across different food applications.

2.3 Dry Fractionation of Proteins

2.3.1 Dry Fractionation Techniques

Several techniques are used in dry fractionation: air classification, sieving, and electrostatic separation. In air classification, particles are separated based on their aerodynamic behavior, which is influenced by their size and density. Based on particle size, two fractions, coarse and fine, are obtained. Most often, centrifugal air classifiers are used in this regard (Fernando, 2021).

Electrostatic separation is a modern method for separating protein-starch mixtures based on particle ionizability. Proteins are more ionizable due to the presence of ionizable groups in their amino acid residues, N-terminus, and C-terminus, whereas carbohydrates lack ionizable groups in their structure (Assatory et al., 2019). Triboelectric separation is a specialized electrostatic separation technique, where the particles are charged by contact or friction, and subsequently subjected to an electric field for separation (Zhao et al., 2024). Various sieving methods are also used to separate protein fractions, such as stacking sieves with varying mesh sizes (Fernando, 2021) or air-jet sieving (Opazo-Navarrete et al., 2018).

2.3.2 Air Classification

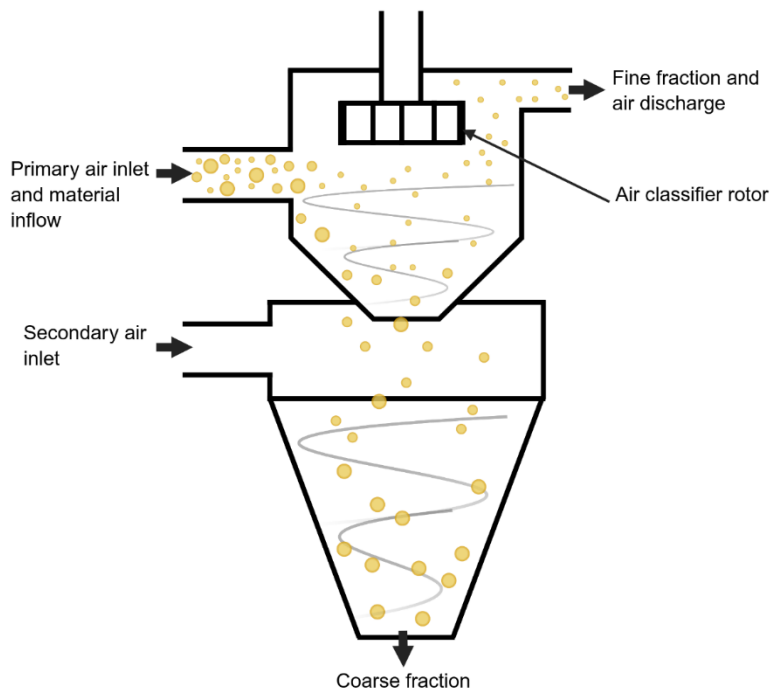


Figure 2.1: Mechanism of cyclone air classifier adapted from (Rempel et al., 2020)

2.3.2.1 Theory and Principles of Air Classification

Air classification is often performed in combination with dry milling. The main aim of dry milling is to separate cellular components according to the type of grain used. In starch-rich legumes such as lentils, peas, etc., proteins are to be released from the starch matrices, whereas in legumes such as soy and lupin, proteins are disentangled from structures of non-starch polysaccharides and fibrous components (Tabatabaei et al., 2023). The efficiency of milling is influenced by numerous factors, such as grain type, cultivar, and mill type (Schutyser et al., 2025). The milled material is then subjected to air currents, which separate the particles based on their physical and chemical properties.

The air classifier consists of several components, including cyclone housing, in which the internal fan introduces a spiraling air current needed for the separation (**Figure 1**). The spiraling air creates centrifugal forces that push particles outward, causing them to collide with the casing. In addition, gravitational forces acting upon the particles cause them to fall into the coarse cyclone, forming the coarse fraction. At the same time, centripetal friction drag acts on the particles, pulling them inward toward the separating rotor. These particles are carried out of the cyclone housing into a secondary cyclone, where they form the fine fraction. When centrifugal and centripetal forces are balanced, particles have an equal likelihood of being classified into either the coarse or fine fraction. The particle size at this point is known as the cut point/ cut size. Separating rotor speed and the air stream rate controls the velocity of the spiraling air, thereby determining the magnitudes of the centrifugal and centripetal forces acting on the particles (Rempel et al., 2020).

2.3.2.2 Factors influencing the air classification efficiency

Several variables can be modulated to optimize the air classification process and achieve maximum efficiency. Among many other factors, milling gains prominence because it has a significant impact

on the detachment of starch and protein bodies, which, in turn, influence separation efficiency (Tagle-Freire et al., 2025). The type of mill and milling speed are important factors that influence subsequent steps in air classification (Fernando, 2021). However, excessive milling can have a detrimental impact on the air classification through effects such as causing poor flow behavior due to stronger van der Waals forces occurring due to the higher surface area (Dijkink et al., 2007) and damaged starch particles entering the fine fraction due to its smaller particle size, thus reducing the purity of the fine fractions (Pelgrom et al., 2014).

Several factors associated with the air classification process determine its efficiency. Among these, the prominent factor is the air classifier wheel speed. Because of its direct relationship with the cut point, the air classification wheel speed governs protein separation into the fine fraction, thereby affecting fractionation efficiency and the chemical composition of the resulting fractions (Denmud et al., 2019). This effect was observed in many studies reporting enhanced enrichment at higher air classifier wheel speeds across a wide range of legumes, indicating its universal applicability (Tabatabaei et al., 2023). In addition, the cut size is influenced by several other processing parameters, such as feed rate and air inlet velocity. Denmud et al. (2019) found that decreasing the air inlet velocity and increasing the feed rate can enhance protein separation into the fine fraction by influencing the cut size. Usage of flowability aids has also been explored as an effective strategy to influence the air classification process. It is expected that these flowability aids adhere to the powder particles and reduce interparticle attraction, thereby increasing the interparticle distance (Müller et al., 2008).

In addition, incorporating various pre-treatments, such as drying, dehulling, soaking, and freezing, can affect air classification efficiency by altering the composition. Politiek et al. (2023) recorded that the moisture contents between 30-70% of the feed material avoided any negative effects

towards the milling and air classification performance, yet an increase in moisture content during storage caused a lack of performance. This is likely caused by poor dispersibility and flowability in the presence of moisture, as the powder particles tend to aggregate. Dehulling also has a significant impact on the air classification efficiency, especially altering the composition of the fractions, mainly due to the changes in the fiber and polyphenolic compounds concentrated in the hulls (Saldanha do Carmo et al., 2022). Reducing the oil content in seeds through defatting before air classification has also yielded better results, possibly due to improved particle dispersibility, which encourages better separation (Dijkink et al., 2007).

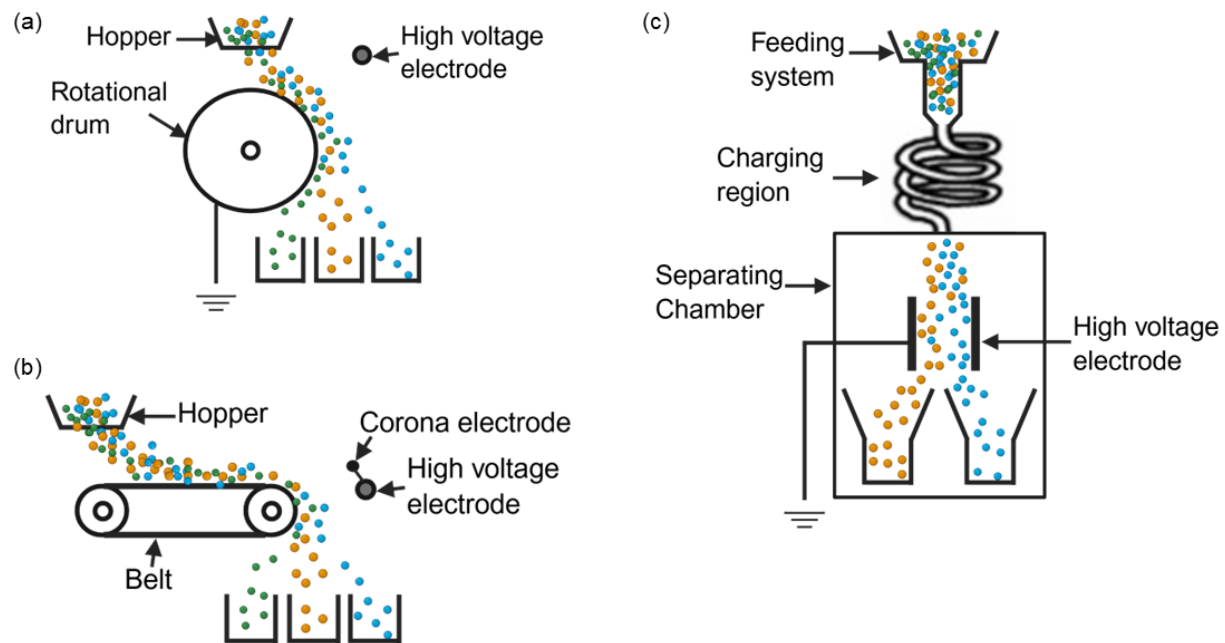
2.3.3 Electrostatic Separation

2.3.3.1 Theory and Principles of Electrostatic Separation

In contrast to the air classification, this technology focuses on the differences in the dielectric properties of protein particles (Assatory et al., 2019). In most fractionation techniques, including air classification, the difference in particle size between components is used as the basis for separation. However, separation efficiency is consistently compromised by the similar aerodynamic behavior of starch and protein particles, as well as by the presence of other components such as damaged starch (Zhu et al., 2021). Therefore, electrostatic separation has emerged as a dry fractionation technique that reduces reliance on particle size and density by exploiting differences in surface charging behavior. This method subjects the particles to triboelectric charging, and based on the triboelectric properties of particles, such as polarity and magnitude, particles are separated in an electric field (Khazaei et al., 2019). Proteins can be charged to a greater extent due to the presence of ionizable groups in their amino acid residues, N-terminus, and C-terminus, while starch and carbohydrates cannot be imparted with a large extent

of charges due to the lack of ionizable groups in their structures (Tabatabaei et al., 2023). Thus, electrostatic protein separation exploits this property.

Three main types of electrostatic separators can be identified, namely, induction charging, corona discharge, and tribocharging (Zhu et al., 2021). Among these methods, induction charging and corona discharging involve passing the material through the discharge area on a conveyor. Charged particles leave the conveyor quickly, while the non-conducting material tends to remain in the conveyor or slowly falls out under the influence of gravity (Mayer-Laigle et al., 2018). Tribocharging separators are considered the most suitable for pulverized plant materials, in which particles are carried by airflow into the pipeline tribocharging unit. Particles are charged upon collision with the inner wall of the pipeline and separated in the separation chamber under the



influence of an electric field (Matsusaka et al., 2010).

Figure 2.2: Different electrostatic separators (a) Induction charging system (b) Corona discharge system (c) Tribocharging system (adopted from (Zhu et al., 2021))

2.3.3.2 Factors influencing electrostatic separation efficiency

Various strategies can enhance the efficiency of electrostatic separation, especially by promoting greater particle dispersibility. Particle size is a critical factor in this regard. Milling is a commonly used pretreatment that reduces particle size, thereby increasing surface area and enabling more charges on particle surfaces (Basset et al., 2016). However, excessive milling may induce aggregation of charged particles, hindering their separation under an electric field as their charges neutralize (Wang et al., 2015). In addition, the type of mill affects the protein separation efficiency of triboelectric separators, as different milling mechanisms have distinct effects on starch and protein disentanglement and on the resulting particle size. This effect was observed in the study by Vitelli et al. (2020), which found that hammer milling yields lower separation efficiency than pin milling due to the larger average particle size. The flour's moisture content is another essential factor that determines separation efficiency. It is important to maintain the feed material's moisture content at a lower level to ensure proper particle flowability and charge conductivity (Wang et al., 2014).

The tribocharging phenomenon relies on collisions between particles and the inner wall of the tribopipe. Therefore, the internal wall material of the tribopipe also plays an integral part in the separation. The selection of tribopipe material should be based on its position in the triboelectric sequence. Generally, it is accepted that proteins readily acquire a positive charge by donating electrons, so materials at the negative end of the sequence are chosen for plant protein separators (Zhu et al., 2021). A study by Xing et al. (2021) demonstrated improved protein separation with PTFE. Some studies by Landauer & Foerst (2018) and Tabatabaei et al. (2016) indicate that materials at the positive end of the sequence, such as nylon, aluminum, or stainless steel, can also impart a strong charge to protein particles, thereby improving separation efficiency. In addition,

designs that extend the residence time within the tribopipe or increase its length can enhance collision probability, thereby allowing particles to acquire greater charge and separate with greater efficiency (Tabatabaei et al., 2016). The gas flow rate is another factor influencing the tribocharging process, as it governs particle-wall contact and collisional impacts. However, the gas flow rate must not exceed optimal conditions, as otherwise the particles may overcharge and aggregate, obstructing separation (Gargari & Tabatabaei, 2024). Similarly, collisions are affected by the concentration of the materials in the feed and are generally favored by a dilute phase, as it allows greater particle dispersibility and an increased likelihood of collisions (Zhu et al., 2021).

Characteristics of the electric field can also be effectively manipulated to enhance electrostatic separation efficiency. The separation chamber configuration can be horizontal or vertical, and horizontal models are expected to have lower separation efficiency due to gravitational force interference (Landauer & Foerst, 2018). It has also been found that applying an electromagnetic field that exerts the Lorentz force (Gao et al., 2019) can enhance charged-particle separation and is also applicable to protein separation. At the same time, the coverage of electrodes by sample particles over time is a challenge that must be addressed to maintain adequate separation performance in triboelectric separators (Tabatabaei et al., 2017). This effect can be mitigated by increasing the electrode spacing (Zhu et al., 2021).

2.3.4 Sieving

Sieving is a particle-size-based separation method similar to air classification. However, owing to its simplicity and familiarity, the scientific principles of sieving are largely overlooked (Liu, 2009). At the same time, sieving performance is highly affected by flour properties and composition, as the particle sizes of different components differ within the flour, making it a heterogeneous blend (Wang & Flores, 2000). The mechanism used for sieving is called a sieving cascade, in which

sieves of decreasing mesh size are stacked on top of each other and mounted on a device that vibrates the sieve set, facilitating particle movement through the sieves via mechanical shaking (Prume et al., 2021). In addition, reverse sieving can be employed, in which a single sieve is used at each step, and the ground material is sieved from the fine to the coarse fraction by subjecting it to several sieving steps (Siliveru & Ambrose, 2021). Despite the method used, the biggest challenging factor is to avoid sieve blinding, the phenomenon of blocking the sieving mesh as the particles are retained within the pores, obstructing any further movement of particles through the mesh, thus hindering the separation. The patterns formed in the mesh due to blinding, along with their associated dynamics, can be explained using percolation theory (Wilkinson & Davies, 1989).

Two types of particle movement accompany the sieving process over and across the sieve mesh: horizontal and vertical. Horizontal movements loosen the larger particles and open them for the finer particles to pass through the mesh, whereas vertical movements provide agitation and mixing, facilitating the redeposition of articles on the mesh (Liu, 2009). However, horizontal movements tend to block the sieve due to the deposition of larger particles, hindering proper separation. Tapping can be used as a strategy to reduce sieve blinding by promoting vertical motion and overcoming the blocking of the mesh by larger particles (Siliveru & Ambrose, 2021). It is essential to maintain a small particle size during sieving; plant material is often milled before sieving. Milling has a significant impact on sieving efficiency because it can change particle size and shape, which govern the particle size distribution of the sieved fractions (Roberts & Beddow, 1968).

2.3.5 Comparison between different methods

Each dry fractionation method has its own advantages and disadvantages, which largely determine its range of applications and suitability for industrial setups (**Table 1**).

Table 1: Comparison between different dry fractionation methods

Technique	Advantages	Disadvantages	Reference
Air Classification	• Solvent-free and environmentally friendly • Preserves native protein structure due to minimal thermal damage • Scalable and industrially established • Cost-effective with low energy consumption • Suitable for protein–starch separation in legumes and cereals	• Limited separation efficiency for particles with similar size and density • Requires precise control of milling and airflow conditions • Lower protein purity compared to wet fractionation	(Schutyser & van der Goot, 2011) (Schutyser et al., 2015) (Assatory et al., 2019) (Fernando, 2021a) (Tabatabaei et al., 2016)
Electrostatic Separation	• High selectivity between protein- and starch-rich fractions • Dry, solvent-free process • Can achieve higher protein purity than air classification alone • Complements air classification	• Sensitive to humidity and particle surface properties • Requires pre-treatment (fine milling, drying) • More complex equipment and higher capital cost • Lower throughput compared to air classification	(Assatory et al., 2019) (Zhu et al., 2021) (J. Wang, de Wit, et al., 2015)

Sieving	• Simple, low-cost operation • Easy to implement at lab and industrial scale • No energy-intensive equipment required	• Limited separation efficiency • Ineffective when particle size distributions overlap • Does not selectively enrich protein fractions • Often used only as a pre- or post-processing step	(Schutyser & van der Goot, 2011)
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These methods are also often compared and contrasted with wet fractionation methods of protein extraction to assess their suitability for the specific application. Therefore, this section examines the advantages and limitations of each method, providing a comprehensive overview of their applicability across various food-processing applications.

Dry fractionation is often associated with its ability to preserve the native structure of proteins, unlike wet extraction, which results in considerable denaturation due to exposure to harsh temperature and pH conditions during extraction (Pulivarthi et al., 2025). Wet extraction typically involves extreme pH adjustments, high ionic strength, organic solvents, and thermal treatments to solubilize and precipitate proteins, which can induce unfolding, denaturation, aggregation, and chemical modifications such as deamidation or oxidation (Akyüz et al., 2024). These affect non-covalent interactions that stabilize the native protein structure, leading to denaturation and, in most cases, unfavorable changes in the protein structure. In contrast, during dry fractionation, proteins remain largely in their native, insoluble matrix and are not exposed to drastic chemical or thermal stresses; their secondary and tertiary structures are retained with minimal degradation. As a result,

dry-fractionated protein concentrates often exhibit higher structural integrity, which can facilitate more predictable techno-functional and nutritional properties (Schutyser & van der Goot, 2011).

Among dry fractionation methods, air classification is often appreciated for its low energy requirements from a sustainability perspective. Calculating the energy requirements of air classification and wet extraction, Schutyser & van der Goot (2011) found a clear reduction in energy consumption in milling and air classification compared to wet extraction. The main energy consumption components identified in the wet extraction were mixing water and flour and evaporation to obtain the dry product. The power dissipated by the airflow and the rotation of the air classifier wheel are the energy components associated with dry fractionation. Accordingly, it was evident that the energy and power requirements for wet extraction largely exceed those of dry fractionation. Similarly, Schutyser et al. (2015) plotted a Sankey diagram of pea protein extraction via air classification and wet extraction, showing that protein recovery per unit of energy consumed is significantly higher with air classification. An added advantage of choosing air classification for dry fractionation is that it is accredited for producing organic food products, and its declaration does not require E-numbers (Schutyser et al., 2015). Thus, air classification is increasingly used in response to the global trend toward clean-label foods. Air classification is also considered a scalable technology owing to its simplicity and low resource and energy requirements (Assatory et al., 2019).

One of the major drawbacks of air classification is the lower protein purity achieved compared to wet protein extraction. However, the presence of oils, fiber, and starch can also be beneficial in various food applications, with implications for the food product's functional and nutritional properties (Schutyser & van der Goot, 2011). The movement of coarse and fine particles to the other fraction rather than the designated fraction is mainly caused by random air turbulence and

the collisions between the particles (Fernando, 2021). At the same time, air classification has proven inefficient at separating particles with similar size and density, thereby reducing fractionation efficiency (Tabatabaei et al., 2016). This is especially apparent when the extent of starch damage is high following milling, as the particle size of starch decreases, their tendency to enter the fine fraction increases, thereby reducing the protein purity of the fine fraction (Pelgrom et al., 2013). It is also essential that oil-rich seeds undergo defatting prior to air classification, as oil will reduce particle dispersibility in the cyclone, thereby affecting separation (Pelgrom, Boom, et al., 2015).

On the other hand, electrostatic separation helps overcome some limitations associated with air classification by reducing dependence on particle size and density. Therefore, this method is particularly useful when the particles are similar in size and density (Assatory et al., 2019). It is also known for its energy efficiency, as studies report lower energy consumption for electrostatic separation than air classification (Zhu et al., 2021). Electrostatic separation can also be effectively coupled with other separation techniques, such as ultrafine milling and sieving, to obtain better protein purity. However, electrostatic separation has a limited ability to separate starch and protein, as they receive similar polar charges during tribocharging, a major disadvantage of the method (Wang, et al., 2015).

Sieving is a very simple separation mechanism with comparatively lower energy consumption. Sieving is often characterized by several drawbacks, namely lower separation efficiency and sieve blocking, which can restrict separation (Schutyser & van der Goot, 2011). Therefore, it is frequently used as a pretreatment prior to electrostatic separation or air classification to enhance separation efficiency.

Accordingly, air classification is one of the most efficient dry fractionation techniques for protein enrichment, owing to its simplicity, scalability, and preservation of native protein functionality. It offers the comparative advantages of improved protein separation efficiency, fewer processing steps, lower energy and resource requirements, and a lower environmental footprint. Additionally, air classification is highly adaptable to industrial-scale operations and allows selective enrichment of protein-rich fine fractions while maintaining the integrity of starch and fiber components. For these reasons, air classification was selected in this study as a sustainable and industry-relevant approach for producing protein-enriched fractions suitable for subsequent functional modification

2.4 Strategies for Enhancing the Efficiency of Air Classification

2.4.1 Factors used to measure the optimization of air classification

Various factors can be used to evaluate the effectiveness of strategies to enhance the efficiency of the air classification process. Some of these factors concern the performance of the air classifier, whereas others concern the composition and functionality of the resulting fractions. In terms of performance parameters, this review primarily focuses on protein yield and separation efficiency. Protein yield is defined as the mass percentage of the original flour that is recovered in the protein-rich fine fraction. Protein Separation Efficiency is defined as the percentage of the total protein in the original flour that is recovered in the protein-rich fine fraction (Schutyser & van der Goot, 2011). Compositional factors can vary widely, but protein and starch content are the main characteristics assessed in this review. Many researchers identify a group of techno-functional properties to assess the protein concentrates, namely water-holding capacity, oil-holding capacity, foaming capacity and stability, emulsion capacity and stability, and gelation ability (Tabatabaei et al., 2023). This section of the literature review has taken all these properties into account whenever the data is available.

2.4.2 Air classifier wheel speed

The air classifier wheel speed is the factor most extensively studied in relation to air classification efficiency. Several studies have been conducted to assess the impact of air classifier wheel speed on the protein separation in different types of flour, mung bean, yellow pea, and cowpea (Schlangen et al., 2022); field peas (Wang & Maximiuk, 2019; Pelgrom et al., 2013); fava bean (Oluwajuyitan & Aluko, 2025); barley (Silventoinen et al., 2018); and brown lentils (Funke, Loeffler, et al., 2022). Most of these researchers have focused on understanding how wheel speed affects the protein yield, protein enrichment, and starch enrichment.

The protein content of the fine fraction increased with increasing classifier wheel speed. This trend was accompanied by a reduction in starch content at higher speeds, likely due to fewer starch granules in the fine fraction. As the classifier wheel speed increases, the particle size that can pass through decreases, thus allowing only smaller particles, such as protein bodies, to pass through the classifier wheel (Pelgrom et al., 2013; Schlangen et al., 2022). A similar trend in protein content was observed for field peas by Wang & Maximiuk (2019) at air classifier wheel speeds of 2500-4500 rpm. However, beyond 4500 rpm, the protein content in the fine fraction decreased while the protein content increased in the coarse fractions. These results contradict the findings of Schlangen et al., who recorded a rise in the fine fraction's protein content as wheel speed increased above 8000 rpm for the same seed material, peas. This contradiction could be affected by the type of air classifier used (the ATP50 air classifier by Schlangen et al. and the NETZSCH-CONDUX classifier by Wang & Maximiuk) or by the variety of field peas used. Similarly, in fava bean fine fractions, protein content increased as the air classifier wheel speed increased from 20 to 35 Hz (Oluwajuyitan & Aluko, 2025). A pattern similar to protein enrichment was observed in starch enrichment, dietary fiber, and ash content by Wang & Maximiuk (2019) as wheel speed increased

from 2500–4500 rpm to above 4500 rpm. The fiber, crude fat, and ash content results from the study conducted by Oluwajuyitan & Aluko (2025) for fava beans also followed the same pattern.

The air classifier wheel speed has also significantly affected the composition of the obtained fractions. It is common to see a decrease in protein yield as protein content increases. This was apparent for all the materials analyzed by Schlangen et al. (2022); mung bean, cowpea, and yellow pea, and by Pulivarthi et al. (2023) for red lentils. For barley, Silventoinen et al. (2018) also observed this decrease it was smaller than the values recorded by other studies, thus indicating a higher protein separation efficiency. Wang & Maximiuk (2019) also observed a decrease in the fine fraction yield and an increase in the coarse fraction yield for field peas as the classifier wheel speed increased from 2,500 to 4,500 rpm. However, the opposite effect was observed when the speed was increased further beyond 4500 rpm. The findings of Oluwajuyitan & Aluko (2025) were also consistent with the above findings, noting that the coarse fraction mass yield for fava bean flour increased up to 30 Hz wheel speed but decreased beyond 35 Hz. Beyond 35 Hz, separation efficiency decreased, resulting in a higher fine-fraction yield.

The air classifier wheel speed also significantly affects particle size. Generally, the fine fraction's particle size decreases with the increasing wheel speed. However, some authors suggest that the level of reduction depends on the seed material. Schlangen et al. (2022) noticed that the D[4,3] value (Volume-Weighted Mean Diameter) related to particle size significantly decreased irrespective of the material as the speed increased, yet the value for cowpea was higher than that of mung bean and yellow pea. Similar results were recorded for fava beans (Oluwajuyitan & Aluko, 2025), red lentils (Pulivarthi et al., 2023), and pea flour (Pelgrom et al., 2013).

The effect of different air classifier wheel speeds on the techno-functional properties of faba bean fine and coarse fractions was assessed by Oluwajuyitan & Aluko (2025). No clear trends in protein

solubility were identified; however, the fractions produced at 35 Hz showed the least susceptibility to heat-induced coagulation at pH (Oluwajuyitan & Aluko, 2025). Both Funke, Boom, et al. (2022) and Oluwajuyitan & Aluko (2025) reported smaller oil droplet sizes during emulsion formation for fine fractions produced at higher classifier wheel speeds, indicating improved emulsifying properties. This enhancement is likely due to the higher protein content of these fractions. In contrast, air classifier wheel speed did not significantly affect water-holding capacity (Oluwajuyitan & Aluko, 2025). At higher wheel speeds, the coarse fractions exhibited greater gelling capacity than the fine fractions.

Among the various factors studied, the effect of air classifier speed has received comparatively more attention in the existing literature. However, its influence on the functional properties of protein concentrates remains underexplored, particularly across diverse plant protein sources. This gap highlights the need for future research, as understanding the impact of classifier speed is crucial for advancing product development and formulation strategies. Additionally, analyzing the secondary structure of protein fractions could provide valuable insights to enhance the functionality and market potential of protein concentrates, especially compared with traditionally produced wet-extracted isolates. The proposed research aims to address these critical gaps by examining the functional and structural characteristics of protein concentrates derived from lentil flour, thereby advancing scientific knowledge and applications in this area.

2.4.3 Flowability aids

Flowability aids are another technique used to enhance the effectiveness of air classification by improving the powder's dispersibility. Aerosil and starch are common flowability aids used in the air classification process. In Aerosil, finely powdered silica particles bind to larger particles, increasing the distance between them. This reduces interactions between these large particles and,

consequently, lump formation (Pelgrom et al., 2014). The effect of Aerosil on the air classification efficiency has been studied by Pelgrom et al. (2014), Silventoinen et al. (2018), and Silventoinen et al. (2025) for lupine, barley, and Bambara groundnut, respectively. In contrast, the effect of potato starch on lupin dry fractionation was studied by Pelgrom et al. (2014).

The impact of adding flowability aids is primarily assessed by measuring flour dispersibility. Accordingly, it was found that the dispersibility of lupine flour was enhanced by Aerosil and potato starch at pressures below 250 kPa. The authors suggested that Aerosil increased the distance between the lupine particles, thereby reducing the likelihood of van der Waals forces. Similarly, the flowability of Bambara groundnut flour also increased with the addition of the flow aid (Silventoinen et al., 2025). Potato starch acts as a carrier for the lupine particles, as they adhere to the starch granules, thereby enhancing their dispersibility (Pelgrom et al., 2014).

Although flowability aids improved flour flowability, they slightly reduced the protein content of the lupine fine fraction. This reduction may be attributed to the possibility that the flowability aids also promoted the separation of smaller non-protein compounds into the fine fraction (Pelgrom et al., 2014). In contrast, Silventoinen et al. (2018) reported a significant increase in the protein content of the fine fraction following the air classification of barley flour. In both studies, however, the addition of a flowability aid increased the yield of the fine fraction, thereby improving overall protein separation efficiency (Pelgrom et al., 2014). Silventoinen et al. (2025) also observed a doubling of the fine fraction yield upon the addition of 0.5% Aerosil to the milled Bambara groundnut before air classification, and a slight increase in the fine fractions' protein content. The addition of starch as a flowability aid produced contrasting results, where the fine fraction yield was reduced. This was attributed to the fact that the lupine particles attached to the potato starch granules, which were carried away into the coarse fraction during the air classification (Pelgrom

et al., 2014). Interestingly, a pronounced positive effect on starch enrichment was not observed in Bambara groundnut when Aerosil was used as a flowability aid (Silventoinen et al., 2025).

The water- and oil-holding capacities of Bambara groundnut fine fractions changed when a flowability aid was used during air classification. The water-holding capacity had decreased, and the oil-holding capacity had increased compared with the native flour (Silventoinen et al., 2025). Although the effect of flowability aids on air classification parameters and composition has been studied comprehensively in previous research, findings on their impact on the techno-functional properties of protein concentrates remain limited. While some indirect insights can be drawn by examining how variations in protein content within the fine fractions influence techno-functionality, this approach does not provide sufficient or conclusive information. Therefore, a clear research gap exists in this area, underscoring the need for targeted studies to fully understand how flowability aids shape the functional characteristics of protein concentrates.

2.4.4 Re-classification

Re-classification is a strategy employed to enhance the yield and purity of the obtained concentrates through dry fractionation (Schutyser & van der Goot, 2011). This involves subjecting the coarse fractions to repeated milling and air classification cycles (Singh et al., 2024) or subjecting the fine fraction to a second separation cycle (Funke, Boom, et al., 2022). A common trend across these studies is that the authors have mainly focused on assessing the impact of reclassification on starch and protein enrichment patterns.

The effect of re-classification on the protein separation efficiency does not seem to be consistent with the intensity of re-milling or re-classification. Singh et al. (2024a) reported that higher efficiency was observed only in the first two cycles; thereafter, efficiency declined. Similar

observations were made by Funke, Boom, et al. (2022) and Boukraâ et al. (2026) regarding the protein content in the repeatedly classified fine fractions, which can be explained by the tendency to form protein aggregates, leading to larger particles that hinder separation into fine fractions. This aggregation can be induced by van der Waals forces and reduced flowability during the air classification process (Assatory et al., 2019).

Reclassification had a clear effect on the starch content of the fine fractions, with starch levels increasing after each air classification step. This increase may be attributed to starch damage during re-milling and reclassification, which likely reduced starch granule size and enabled their separation into the fine fraction alongside smaller protein particles (Singh et al., 2024a). Repeated classification increased protein enrichment in fine fractions when they were reclassified (Funke et al., 2022) and yielded a higher combined protein separation when coarse fractions were reclassified (Singh et al., 2024a). The protein content in each fine fraction decreased with the intensity of reclassification, which could again be attributed to the effect of starch granular size (Rempel et al., 2019). However, these efficiencies depend on the type and the variety of the seed. Reductions in the crude fat content in fine fractions and separation of dietary fiber into coarse fractions were observed with repeated classification (Singh et al., 2024; Rempel et al., 2019).

As with other influencing factors, the available information on the effect of re-classification is mainly limited to air classification parameters and protein enrichment levels. Moreover, there is a noticeable lack of studies specifically focused on the re-classification process itself. Reclassification has significant potential to enhance the purity of protein concentrates, a key limitation when comparing them to protein isolates. Therefore, it is essential to conduct further research in this area and gather detailed data from various sources to better understand and optimize this process.

2.4.5 Dehulling as a Pre-treatment

Dehulling is the process of removing the seed coat (hull) from seeds. The level of adherence of the hulls may vary by pulse type; therefore, they are associated with different levels of difficulty in removal. Tightly adhering seed coats are removed by abrasive-type dehullers, whereas loose hulls are removed by attrition-type dehullers like hammer mills (Fernando, 2021). Based on the available literature, dehulling of peas and faba beans can be performed using a burr mill, a type of abrasive mill (Saldanha do Carmo et al., 2020). In some cereals, such as barley, hull-less varieties have been developed and can be obtained directly without a dehulling step (Singh et al., 2024). Dehulling is expected to have a clear effect on most techno-functional, compositional, and structural characteristics of the protein concentrates, primarily by affecting their fiber content. However, available studies are mostly limited to the impact of dehulling on air classification parameters and enrichment levels.

The protein separation efficiency in peas increased after dehulling. The suggested explanations for this phenomenon were possible adherence of protein particles to the hull fragments or the impact of the hulls on the material's flow properties during air classification. A similar effect was not observed with faba beans, indicating that the effect depends on the material as well (Saldanha do Carmo et al., 2020). Despite recording a significantly higher protein content in the hull-less barley variety, Singh et al. (2024) do not attribute it to the presence or absence of hulls.

In terms of chemical composition, it was observed that the transfer of starch to the coarse fraction in both peas and faba beans was enhanced by dehulling. In contrast, the non-starch polysaccharides (NSP) in the fine fractions were enhanced after dehulling. Thus, it is observed that NSP behaves similarly to the proteins during dry fractionation (Saldanha do Carmo et al., 2020).

Dehulling also influenced several techno-functional properties of pea and faba bean concentrates. In faba bean fine fractions, protein solubility at pH 10 decreased after dehulling, whereas no significant changes were observed at other pH values. This effect may be related to the influence of alkaline pH on the NSP content in the fine fractions (Saldanha do Carmo et al., 2022). The water-holding capacity of the fine fractions was reduced upon dehulling, as the fiber content decreased with the removal of the hulls (Saldanha do Carmo et al., 2021). However, no significant difference in oil-holding capacity was observed between peas and faba beans. The emulsifying capacity was improved upon dehulling. Dehulling increased the emulsion capacity of the concentrates slightly, yet had no significant impact on the emulsion stability (Saldanha do Carmo et al., 2020).

Only a limited number of studies have investigated the effect of dehulling on the efficiency of the dry fractionation process ((Saldanha do Carmo et al., 2022; Saldanha do Carmo et al., 2020; Singh et al., 2024) underscoring the need for further research in this area. It is clear that the impact of dehulling varies with the seed material used, which further enhances the requirement for systematic evaluation.

2.4.6 Milling as a Pre-treatment

Protein enrichment during air classification relies on the size difference between protein particles and other components, such as fiber and starch. Milling is an important processing step before air classification, as it allows the detachment of starch and protein bodies, facilitating their separation during classification. Thus, milling parameters should be carefully optimized to ensure maximum separation (Pelgrom, Boom, et al., 2015). Several types of milling machines are used in industry and are classified by the mechanism they use. Impact mill (Pelgrom et al., 2015; Pelgrom et al., 2013), cutting mill (Saldanha do Carmo et al., 2020), and fluidized-bed jet mill (Pelgrom et al.,

2013) are examples reported in the literature. Pin mills (Wang & Maximiuk, 2019) and hammer mills are types of impact mills used frequently in dry fractionation. Pin mills consist of two discs that rotate at different speeds, and the fed seeds are directed towards the stationary disc at the center. Centrifugal force causes collisions between the seeds and the pins mounted on the stationary disc, resulting in grinding. In hammer mills, the impact force is generated by fixed or swinging hammers mounted inside a cylindrical casing. Seeds are subjected to the grinding action as they pass between the hammers and the casing. Roller milling is another type of mill, yet it is used less frequently in the dry fractionation process. More advanced milling techniques, such as cryogenic milling for heat-sensitive materials and ultrafine classifier milling, which combines milling with particle size classification, have also been developed (Pulivarthi et al., 2023). In milling experiments, several variables can be tested, including mill speed, internal classifier wheel speed, airflow, and feed rate. However, most studies have focused on assessing the impact of changing the internal classifier wheel speed while keeping other factors constant.

Protein separation efficiency is expected to improve with milling due to changes in particle-size distribution. This effect was clearly observed in twice-milled pea samples, which showed a marked improvement in protein separation. In faba bean samples, the effect was less pronounced but still positive. The authors attributed the effect on the pea samples to reduced adhesion of protein bodies to starch granules as particle size decreased with increasing milling intensity (Saldanha do Carmo et al., 2020). However, the literature also suggests that a one-step milling procedure for peas is adequate to achieve higher protein separation efficiency, given the particle sizes of protein bodies, starch granules, and whole cells (Pelgrom et al., 2013). At a classifier wheel speed of 8000 rpm, separation efficiency is predicted to decline because starch granules may fragment into particles smaller than the protein bodies. This trend was confirmed by Pelgrom et al. (2013). Similarly, yield

decreased at higher wheel speeds, regardless of whether an impact mill or a jet mill was used. This reduction was attributed to weakened flow behavior resulting from increased particle surface area and stronger van der Waals interactions (Pelgrom et al., 2014). It was reported that operating the pin mill with the internal classifier at higher speeds resulted in a narrower particle-size distribution and a lower average particle size (Pelgrom, Boom, et al., 2015). The reduction in particle size was also observed by Pelgrom et al. (2014) for lupin proteins.

Additionally, the morphology of starch granules was affected by mill type at higher classification speeds. Jet milling produced smaller starch particles with greater starch damage. This affects the quality of the starch and the separation of the proteins (Pelgrom et al., 2013). Milling can influence the techno-functional properties of protein concentrates by altering particle size. However, these potential impacts remain largely underexplored, as existing studies have not specifically focused on this aspect. Consequently, a clear research gap exists, highlighting the need for further investigation into the role of milling in shaping the functional attributes of plant protein concentrates.

2.4.7 Other Pre-treatments

Several pretreatments have been evaluated for their impact on the air classification process. Most of these techniques affect the separation of protein bodies from other cellular components. The addition of moisture has positively influenced detachment in some materials, such as hard wheat, whereas the opposite effect is evident in faba beans, peas, etc. Furthermore, adding moisture increased the mechanical energy requirement during milling as the seeds became more ductile. Freezing also induces damage to cellular structure, thereby increasing the separation of protein particles from other cellular components and improving separation efficiency (Pelgrom, Wang, et al., 2015). In addition, combining air classification with micronization effectively reduced particle

size, thereby enhancing protein enrichment. This effect arises from the intense mechanical forces generated during micronization, which facilitate the detachment of starch granules and protein bodies (Boukraâ et al., 2026).

Defatting, soaking, freezing, and oven drying are among the pre-treatments studied for their impact on air classification. Defatting is the process of reducing fat content to improve separation. It is believed that the flour's fat content reduces its dispersibility and flowability, leading to adhesion to equipment walls. Defatting can be achieved by conventional solvent extraction with organic solvents such as petroleum ether (Pelgrom et al., 2015) or by supercritical carbon dioxide (SC-CO₂) extraction (Silventoinen et al., 2025)

Silventoinen et al. (2025) observed that SC-CO₂ extraction of Bambara groundnut flour improved flowability and reduced the minimum outlet size required for optimal flow from the silo. However, it increased the wall-friction parameters, namely, the friction angle and maximum bulk density, suggesting poor flow behavior of the flour. Defatting the flour using supercritical CO₂ extraction enhanced the positive effects of flowability aids on the mass yield of the fine fraction by simultaneously increasing the overall yield (Silventoinen et al., 2025). Oven drying to reduce moisture enhanced protein separation efficiency by increasing the brittleness of the seed particles (Pelgrom, Wang, et al., 2015).

The effect of defatting on the chemical composition is dependent on the type of seed used. Bambara groundnut subjected to supercritical CO₂ extraction exhibited enhanced contents of protein, starch, and ash in the fine fractions. Lipid and ash enrichment in the fine fractions followed a similar pattern to the protein enrichment, suggesting a close association between the proteins, lipid, and ash in Bambara groundnut seed composition (Silventoinen et al., 2025). In lupin, the fine fraction's protein content increased after defatting, accompanied by a decrease in the fine fraction yield.

Defatting reduces the flour's cohesiveness, thereby enabling better separation of the small particles into the fine fraction. However, there was no significant effect in pea fine fractions (Pelgrom, Wang, et al., 2015). Reducing the moisture content negatively affected the protein content in the fine fraction and milling. Milling at reduced moisture content caused the breakage through the starch and protein bodies rather than between them. This reduced the separation between starch and protein bodies (Pelgrom, Wang, et al., 2015). Due to pre-soaking, a reduction in the fine fractions' protein content and an increase in that of the coarse fractions were observed for pea and lupin flours. This could be due to the effect of soaking on the integrity of the protein bodies, leading to efficient separation during air classification (Pelgrom, Wang, et al., 2015). Regarding functional properties, supercritical CO₂ extraction slightly reduced protein solubility across all pH values. An increase in oil-binding capacity and a decrease in water-binding capacity were observed when the defatted raw material was used to produce the fine fraction. The defatted samples showed a slightly lower pasting temperature, a slightly lower peak viscosity, and significantly lower final viscosity (Silventoinen et al., 2025).

2.4.8 Post-treatments

Some researchers have explored combining different dry fractionation techniques to enhance the protein purity of concentrates and develop products with functional properties comparable to those of wet-extracted protein ingredients. Xing et al. (2020b) attempted to develop a two-step dry fractionation procedure in which air classification was followed by triboelectrostatic separation. During air classification, starch separation occurred, and electrostatic separation effectively separated the fiber from the concentrates. This resulted in fine fractions with higher protein enrichment and purity for peas and lentils. However, the method did not successfully enrich chickpea fine fractions, likely due to the smaller starch granule size.

Solid-state fermentation as a post-treatment was assessed by Xing, Dekker, et al. (2020) as a method for enhancing the compositional and techno-functional properties of chickpea concentrates. It was found that fermentation can increase the fine fraction's protein content while reducing certain antinutrient compounds. The most noteworthy impact was the reduction in phytic acid, a compound that reduces protein content in protein ingredients. In addition to altering the composition, fermentation also enhanced the water-holding capacity of chickpea protein concentrates. In contrast to the above, foaming properties decreased during fermentation.

Bühler et al. (2020) investigated the potential of dry heat treatment to alter the functional properties of faba bean protein concentrates, specifically water-holding capacity. Accordingly, it was recorded that the water-holding capacity of the concentrates increased upon the application of dry heat. A reduction in the protein solubility was also observed. Dry heat partially denatured the proteins, leading to increased aggregate formation.

2.4.9 Other strategies

While numerous variables can be adjusted during the air classification process, several remain underexplored in current research. The airflow rate is one factor that has not been adequately studied. De Angelis et al. (2021) studied the impact of using a restriction valve during the fractionation of pea and lentil flour and noted that a tighter restriction valve resulted in a lower airflow rate, and that a lower speed increased protein content but reduced yield and separation efficiency. However, protein separation by dry fractionation of sunflower meal yielded different results: protein content increased with increasing airflow protein separation by dry fractionation of sunflower meal yielded different results: protein content increased with increasing airflow (Banjac et al., 2017).

In summary, the air classification process plays a critical role in separating and concentrating proteins across various industries, particularly in plant protein extraction. This review has highlighted several strategies to enhance the efficiency of the air classification process, including optimizing key parameters, such as air classifier wheel speed, and pre-treatments, such as dehulling and milling. Additionally, the use of flowability aids, re-classification, other pre-treatments, and various post-treatments has been identified as a key factor in improving the air-classification efficiency. These observations were based on their impact on the air classifier's performance and on the protein concentrate's compositional and functional characteristics. Despite progress in understanding these strategies, significant research gaps remain, particularly regarding their impact on various pulse protein concentrate production processes. Future studies should focus on these underexplored areas and develop more refined techniques to improve the process's economic and functional performance. Addressing these gaps will not only improve processing efficiency but also support the growing demand for high-quality plant-based protein concentrates in food and industrial applications.

2.5 Strategies for Enhancing the Functionality of Protein Concentrates

Numerous strategies are employed to enhance the compositional and techno-functional properties of plant protein concentrates. They can be classified according to the principles used. One of the most common ways to classify methods is to categorize them as physical, chemical, or biological.

2.5.1 Physical modification methods

Physical methods to improve protein functionality include heat treatment, high-pressure processing (HPP), ultrasound treatment, extrusion cooking, and cold plasma technology (Akharume et al., 2021). In these methods, proteins undergo denaturation and subsequent rearrangement, thereby altering functional properties such as solubility, water-holding capacity,

emulsifying, and foaming properties. During heat treatment, thermal motion of peptide chains disrupts inter- and intramolecular hydrophobic interactions, electrostatic interactions, hydrogen bonds, and disulfide bonds. Initially, the unfolding is reversible, but later becomes permanent, causing the protein to lose its secondary and tertiary structures. This permanent denaturation exposes the protein's hydrophobic core, enabling the formation of crosslinks through hydrophobic interactions, hydrogen bonding, and disulfide/sulfhydryl bonding (Mession et al., 2015). High-pressure processing also affects proteins by compressing protein cavities and disrupting non-covalent interactions; the resulting conformations enhance protein functionality through distinct mechanisms (Queirós et al., 2018). Ultrasound treatment induces conformational changes in proteins through hydrodynamic shearing from ultrasonic cavitation and thermal heating of protein molecules (O'Sullivan et al., 2017). Cold plasma technology is an emerging field that enables protein modification by inducing structural changes via the energy of reactive species generated by cold plasma. This energy disrupts covalent and hydrogen bonds, leading to structural and functional modifications (Nasrabadi et al., 2021).

2.5.2 Chemical modification methods

Chemical modification methods can also be used for protein modification, but they are less favored due to the growing trend toward clean-label products. These methods primarily rely on the reactivity of protein side chains. Chemical glycosylation is the process of attaching carbohydrate moieties mainly to the amino acid side chain lysine residues or the N-terminus of protein molecules. It can be achieved by manipulating the early stages of the Maillard reaction (Akharume et al., 2021). Acylation is a technique that modifies the protein by transferring an acyl group to the amino or hydroxyl groups of amino acid residues. Succinylation is the acylation that involves a succinyl group, whereas acetylation and maleylation involve an acetyl group and maleic

anhydride, respectively (Xu et al., 2022). Chemical deamidation is another technique that converts amide groups in asparagine and glutamine to carboxylic acid groups, producing aspartic and glutamic acids by releasing ammonia (Li et al., 2010). Phosphorylation modifies the amino acid residues with -NH, -OH, or -SH side groups by attaching a phosphoryl group (Hu et al., 2019). Modification of protein functionality through protein-polyphenol interactions is another chemical modification technique. Through these interactions, protein unfolding occurs, exposing hydrophobic residues and thereby stimulating changes in the protein.

2.5.3 Biological modification methods

In addition to these methods, biological approaches such as enzymatic modification, enzymatic crosslinking, and fermentation can be used effectively to modify proteins. Enzymatic hydrolysis exploits the ability of proteolytic enzymes to cleave the proteins at a site specific to the enzyme. Common proteolytic enzymes include trypsin, pepsin, and papain. Enzymatic crosslinking is achieved using transglutaminase and other oxidative enzymes to improve the textural properties of proteins (Akharume et al., 2021). Thus, a wide variety of protein modification methods are available, and their applications vary depending on the protein ingredients and end-product specifications.

2.6 Cold Plasma technology

Nonthermal protein modification techniques are gaining traction in food processing due to their ability to overcome limitations of conventional thermal treatments. Among these, cold plasma technology stands out as a promising approach that can enhance protein functionality, improve digestibility, and reduce allergenicity. Recognized as a sustainable, environmentally friendly method, cold plasma is considered a green processing technology (Olatunde et al., 2023).

(Quan et al., 2019).

2.6.1 Principle of Cold Plasma

Plasma is the fourth state of matter, achieved by providing energy beyond the phase transition from solid to gas. As the energy supply exceeds a threshold, gaseous molecules are ionized, forming a plasma of ionized particles (Harikrishna et al., 2023). This process generates a range of reactive species, including positive and negative ions, free radicals, neutral and excited molecules, atoms, electrons, and electromagnetic radiation (Bahrami et al., 2022).

Plasma can be broadly classified as thermal or non-thermal based on the energy distribution of its constituent species. In thermal plasma, electrons, ions, and neutral particles are in thermal equilibrium, resulting in extremely high gas temperatures (often several thousand Kelvin) that limit its use to high-temperature industrial applications such as welding and metal processing. In contrast, non-thermal plasma, commonly known as cold plasma, exists in a non-equilibrium state in which electrons are highly energized while the bulk gas temperature remains near ambient conditions. This enables cold plasma to generate reactive oxygen and nitrogen species, UV radiation, and charged particles without causing significant thermal damage. Owing to its low operating temperature and high reactivity, cold plasma has gained considerable attention for applications involving heat-sensitive materials, including food processing, biomedical treatments, and modification of biopolymers such as proteins (Olatunde et al., 2023).

A wide range of cold plasma treatment systems has been developed using various energy sources, including alternating current (high-frequency), direct current, thermal, microwave, radio-frequency, and magnetic fields. Different gas sources have also been used effectively to generate cold plasma, including H, Ne, CF₄, CH₄, NH₃, O₂, He, N₂, Ar, and atmospheric air (Harikrishna et

al., 2023). Accordingly, cold plasma technology can be readily tailored to specific industrial requirements by adjusting the working gas composition and energy input.

2.6.2 Different Cold Plasma Generation Methods

Several non-thermal plasma discharge techniques are employed depending on the underlying discharge mechanism and the desired target response, including the nature and intensity of reactive species generated. These techniques, such as dielectric barrier discharge, plasma jets, corona discharge, and gliding arc systems, vary in their electrical configuration, operating conditions, and interaction with the treated material, allowing the plasma process to be tailored for specific applications such as microbial inactivation, surface modification, or enhancement of functional properties.

2.6.2.1 Dielectric barrier discharge (DBD)

This approach uses a dielectric material positioned between or around two flat metal electrodes, preventing continuous current flow and suppressing spark formation (**Figure 3a**). Cold plasma is produced by introducing an inert or neutral gas into a closed reaction chamber and exposing it to an electric field. Thus, the gas becomes ionized, generating reactive species (Figuerola-Pinochet et al., 2022). Among the two electrodes, one electrode is supplied with high-voltage power while the other remains grounded. It operates across a broad range of gas pressures (typically 10^4 – 10^6 Pa) and frequencies (approximately 0.05–500 kHz), with energy requirements generally between 10 and 100 W (Harikrishna et al., 2023). Several operational parameters, including the applied voltage, gas composition, and electrode gap, influence the efficiency of dielectric barrier discharge. Owing to its uniform plasma generation and scalability, dielectric barrier discharge is particularly well suited to treating large surface areas (Ehlbeck et al., 2011).

2.6.2.2 Corona Discharge Plasma

In this process, plasma is generated at atmospheric pressure through a varying electric field. Corona discharge typically occurs at sharp edges or along thin wires, where a highly non-uniform electric field is established (**Figure 3b**). When the local electric field exceeds the gas's breakdown strength, a weakly ionized plasma with faint luminosity forms. Corona discharge systems can be constructed using relatively simple configurations and do not require complex equipment, resulting in lower installation and maintenance costs. However, a major limitation of this technique is that the plasma treatment is confined to a small region and is often non-uniform (Du et al., 2019). Despite this drawback, corona discharge remains a suitable and widely used option for food sterilization applications.

2.6.2.3 Plasma jet systems

Plasma jet systems consist of two parallel electrodes through which a carrier gas flows at controlled velocities. Plasma jets or plasma plumes are commonly generated at atmospheric pressure using radio-frequency power sources. The excited plasma species are transported out of the discharge region through a small nozzle or orifice near the electrode tip by the flowing carrier gas (**Figure 3c**). Noble gases, such as helium or argon, are typically used at relatively high flow rates (>10 L/min) to sustain a stable plasma jet (Laroussi, 2015). Plasma jets offer the advantage of localized, direct application and are well-suited to treating small or targeted areas. However, despite their suitability for certain biological and medical applications, their use in food processing is limited due to the high operational costs associated with the required gas flow rates (Harikrishna et al., 2023).

2.6.2.4 Microwave plasma discharge

Microwave plasma discharges are generated by a high-frequency electromagnetic field produced by a magnetron, typically operating at 2.45 GHz, rather than by electrodes at different potentials (Chaichumporn et al., 2011). In this process, microwave radiation accelerates electrons, which then collide with gaseous atoms and molecules, producing ions and resulting in a high degree of ionization (**Figure 3d**). The primary advantage of microwave-driven plasma is its electrode-less configuration, which simplifies operation and reduces issues related to electrode erosion, making the system more user-friendly (Ehlbeck et al., 2011). However, a key limitation is the restricted treatment volume, as large areas often require multiple discharge sources for effective decontamination. Consequently, this technique is more commonly applied in high-temperature processing applications (Harikrishna et al., 2023).

2.6.2.5 Gliding arc plasma system

Gliding arc plasma systems are a type of non-thermal plasma discharge that operates at atmospheric pressure and combines characteristics of both thermal and non-thermal plasmas. In this system, an electric arc is initiated between two diverging electrodes and subsequently elongates and glides along the electrodes due to gas flow, leading to partial plasma cooling (**Figure 3e**). As the arc stretches, the gas temperature decreases while maintaining a high level of chemical reactivity through the generation of reactive species (Fridman et al., 1999). Gliding arc plasma is valued for its high energy efficiency and ability to produce large quantities of reactive oxygen and

nitrogen species, making it suitable for applications such as gas treatment, microbial inactivation, and surface modification (Rabinovich et al., 2022).

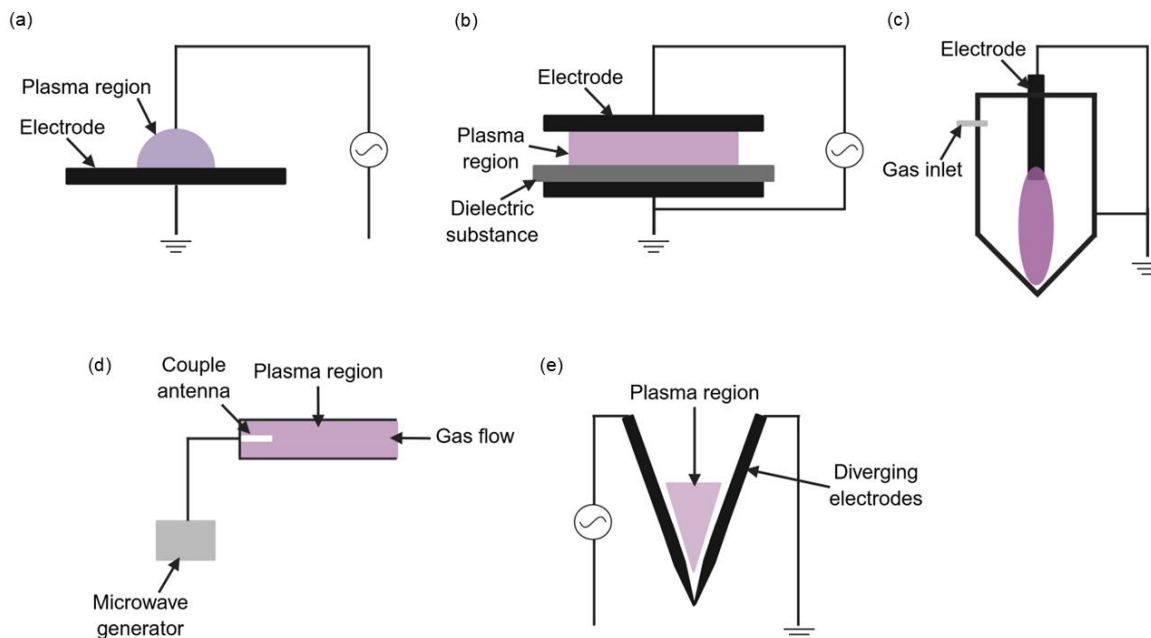


Figure 2.3: Different cold plasma generation methods (a) corona discharge, (b) dielectric barrier discharge, (c) jet plasma, (d) microwave discharge, and (e) gliding arc plasma system, adapted from (Harikrishna et al., 2023)

2.6.3 Chemical Reactivity and Species Generation

Cold plasma has emerged as a versatile technology, with its diverse applications driven by the quantity and composition of reactive species generated during plasma discharge. These reactive species encompass a wide range, including ions, neutrals, radicals, and electromagnetic radiation. Electromagnetic radiation generated during plasma generation plays a significant role in delivering the functional effects anticipated from cold plasma treatment. Within cold plasma systems, these radiations exist as highly energetic photons belonging to the vacuum ultraviolet (VUV) wavelength range (10–200 nm) and the ultraviolet (UV) range (100–400 nm). These high-energy particles play an integral role in the energy transport within the plasma system (Golda et al., 2020). Among these

two types of particles, VUV photons are involved in photoionization and dissociating chemical bonds (Benedikt et al., 2013), inactivate microbial cells (Schneider et al., 2011), induce crosslinking (Hudis & Prescott, 1972), alter the DNA of living cells (Munakata et al., 1991), and cause photolysis in liquid water (Jablonowski et al., 2015). Accordingly, VUV photons have gained much importance in modulating the functional effects of atmospheric cold plasma. In contrast, UV photons are considered more effective in low-pressure plasma, as they are readily absorbed by atmospheric air, limiting their effects during cold plasma treatment (Liu et al., 2020).

Reactive species formed during the plasma generation are highly dependent on the gas composition used. Oxygen gas is often selected as the gas source due to its strong oxidative potential and its ability to generate a diverse range of reactive oxygen species (ROS) during plasma generation. Upon receiving energy, oxygen molecules undergo excitation, dissociation, and ionization, leading to the formation of highly reactive species such as atomic oxygen (O), singlet oxygen ($^1\text{O}_2$), ozone (O_3), superoxide anions, and hydroxyl radicals (Dharini et al., 2023) (**Table2**) These species are particularly effective in microbial inactivation through molecular modifications (Cheng et al., 2020), and oxidative modifications in food macromolecules, which can alter functional properties such as solubility, emulsifying capacity, and water-holding ability through the exposure of polar groups and restructuring of protein conformations (Rout et al., 2025). Compared to inert gases, oxygen provides enhanced chemical reactivity, enabling shorter treatment times and improved process efficiency. In addition, using oxygen as a plasma gas source offers practical advantages: it is readily available and cost-effective, and it decomposes into non-toxic by-products after treatment.

Nitrogen gas is another widely used gas source in cold plasma systems. Upon being subjected to an electric field, nitrogen molecules become excited, ionized, or dissociated, forming reactive

species such as nitric oxide (NO), nitrogen dioxide (NO₂), and other radical species (Dharini et al., 2023) (**Table 2**). Unlike oxygen-based plasma systems, nitrogen-based plasma systems typically produce lower levels of aggressive oxidizing species, reducing the risk of excessive oxidative damage while still enabling microbial inactivation and surface modification. Therefore, nitrogen gas is widely used in plasma systems to modify food macromolecules, such as proteins, thereby altering their functional properties, including solubility, emulsification, and foaming capacity.

Atmospheric air is widely used as a plasma gas source due to its availability, low cost, and ability to generate a complex mixture of reactive oxygen and nitrogen species (RONS) under plasma discharge. Because air is primarily composed of nitrogen and oxygen, air-based plasma systems simultaneously produce reactive species such as ozone, atomic oxygen, hydroxyl radicals, nitric oxide, nitrogen dioxide, and peroxyxynitrite (Dharini et al., 2023) (**Table 2**). In food and protein processing applications, atmospheric air plasma has demonstrated strong antimicrobial function and the ability to induce controlled surface and molecular modifications without excessive heating. The presence of both ROS and RNS enables a broad range of protein interactions, including oxidation, nitration, and alterations in surface charge, which can influence functional properties such as solubility, emulsifying capacity, and water- and oil-holding abilities (Los et al., 2017).

In addition to reactive gases, air, oxygen, and nitrogen, cold plasma systems also extensively use inert gases such as argon, neon, and helium. They offer several advantages over the reactive gases, making them more applicable for food applications. Their chemical inertness and lower ionization potential are among the main reasons inert gases are preferred as plasma gas sources. Due to the lower ionization energy requirement, the ionization power required is lower, thereby reducing operating costs (Vagin et al., 2020). At the same time, more ionized particles and reactive species are produced when inert gases are used because of their lower ionization energies (Dharini et al.,

2023). The absence of strong oxidizing species also reduces the risk of excessive oxidation of proteins, lipids, and other sensitive components, thereby preserving nutritional quality. In addition, many studies have effectively explored the ability to incorporate mixtures of gas plasmas, including inert gases, thereby further enhancing their reactivity and reducing operational costs (Deepak et al., 2018).

Table 2: Properties of reactive species generated by different plasma gas sources

Plasma gas source	Major reactive species generated	Dominant reactivity	Typical role in food/protein applications	References
Oxygen (O ₂)	O•, ¹ O ₂ , O ₃ , •OH, O ₂ ⁻	Strong oxidative	Surface oxidation, microbial inactivation, exposure of hydrophilic groups, and functional property modification	(Dharini et al., 2023) (Yepez et al., 2022)
Nitrogen (N ₂)	N•, NO, NO ₂ , N ₂ ⁺ , excited N ₂ *	Mild oxidative	Controlled protein modification, surface charge alteration, with reduced oxidative damage	(Dharini et al., 2023) (Bradú et al., 2020) (H. Jiang et al., 2022)

Atmospheric air	O•, •OH, O ₃ , NO, NO ₂ , ONOO ⁻	Mixed ROS & RNS	Synergistic antimicrobial action, combined oxidation– nitration, broad functional enhancement	(H. Jiang et al., 2022) (Dharini et al., 2023)
Argon (Ar)	Ar*, Ar ⁺ , secondary ROS/RNS	Physical/secondary interactions	Surface modification, conformational changes, controlled functionality improvement	(Sun et al., 2019) (H. Jiang et al., 2022)
Helium (He)	He*, He ⁺ , secondary ROS/RNS	Physical/secondary interactions	Surface modification, conformational changes, and controlled functionality improvement	(Sun et al., 2019) (H. Jiang et al., 2022)

2.7 Applications of HVACP in Agriculture and Food Processing

2.7.1 Seed treatment and germination enhancement

Cold plasma treatment has emerged as a promising, non-thermal approach for seed treatment and germination enhancement in agricultural applications. Exposure of seeds to cold plasma generates reactive oxygen and nitrogen species (RONS), ultraviolet radiation, and electric fields, which collectively modify the seed surface and surrounding microenvironment without causing thermal damage. One of the primary mechanisms underlying germination enhancement is the alteration of seed coat properties, including increased surface roughness and wettability, which improves water uptake and accelerates imbibition (Benabderrahim et al., 2025) (**Table 3**). Cold plasma treatment has also shown effectiveness in seed decontamination by inactivating pathogenic microorganisms

on seed surfaces, thereby reducing disease pressure during early growth stages (Orlowski et al., 2025). It also promotes seed dormancy breaking, thereby enhancing germination rates across a wide range of seeds, as evidenced by numerous studies (Jiang et al., 2014). In addition, plasma-generated reactive species can stimulate biochemical processes by modulating redox signaling pathways, activating antioxidant enzymes, and influencing phytohormone balance, thereby promoting early metabolic activity essential for germination (Priatama et al., 2022).

However, across various studies, the effect of cold plasma treatment on seed treatment and germination enhancement is highly dependent on several variables. These include the type of cold plasma system, operating conditions, and seed characteristics, such as species physiology and moisture content. (Pauzaite et al., 2018). When applied under optimized conditions, cold plasma treatment can improve germination rate, uniformity, and seedling vigor across a wide range of crop species. Owing to its chemical-free nature, low energy requirements, and environmental sustainability, cold plasma represents an attractive alternative to conventional chemical seed treatments, with significant potential to improve crop development and agricultural productivity.

Table 3: Examples of cold plasma seed treatment studies and germination enhancement

Crop/Species	Cold Plasma Treatment (gas/source & system)	Effects Observed	Reference
Cotton seeds	Dielectric Barrier Discharge plasma	Increase seed water uptake and improve warm-germination, metabolic-chill-test	(de Groot et al., 2018)

	system with Argon	germination, and chilling	
	gas	tolerance in cotton.	
Wheat (<i>Triticum aestivum</i>)	Dielectric Barrier Discharge plasma system with atmospheric air	Enhancement of seed germination, changes in seed surface, water uptake, and contact angle values	(Los et al., 2019)
Radish (<i>Raphanus sativus</i>)	Dielectric Barrier Discharge plasma system with atmospheric air	Increase in seed germination and seedling growth when treated with plasma-activated water (PAW),	(Sivachandiran & Khacef, 2017)
Tomato (<i>Solanum lycopersicum</i>)		Further enhancement when seeds are combinedly treated with PAW and plasma treatment	
Sweet pepper (<i>Capsicum annum</i>)			
Fenugreek (<i>Trigonella foenum-graecum</i>)	Plasma jet system with argon	Improved seed germination rate associated with the seed coat changes, improved wettability, and surface modifications	(Guragain et al., 2024)(Guragain et al., 2024)
Peanut (<i>Arachis hypogaea</i>)	Dielectric Barrier Discharge plasma system with helium	Improvement in germination rate, uniformity of emergence,	(Li et al., 2016)(Li et al., 2016)

		seedling shoot and root dry weights,	
		Enhancement of yield components	
Wheat (<i>Triticum</i> spp.)	Dielectric Barrier Discharge plasma system with helium	Enhanced germination potential, germination rate, plant height, root length, and fresh weight at all growth stages	(Jiang et al., 2014b)(Jiang et al., 2014b)
Soybean (<i>Glycine max</i> L.)	Dielectric Barrier Discharge plasma system with helium	Increased germination and vigor indices, enhanced water uptake, shoot length, shoot dry weight, root length, and root dry weight	(Ling et al., 2014)(Ling et al., 2014)
Soybean cultivars	Dielectric Barrier Discharge plasma system with argon	Enhanced germination potential and rate, improved enzyme activity favoring germination	(Sayahi et al., 2024)(Sayahi et al., 2024)
Brown rice cultivars (<i>Oryza sativa</i> L.)	Dielectric Barrier Discharge plasma system with argon	Increase in germination percentage, root length, and seedling height measurements,	(Yodpitak et al., 2019)(Yodpitak et al., 2019)

		reduced germination time to	
		reach the maximum levels of	
		most phytochemicals.	
Cumin	Dielectric Barrier	Increased germination	(Namjoo et al.,
(<i>Cuminum</i>	Discharge plasma	percentage, germination index,	2022)(Namjoo et al.,
<i>cuminum</i> L.)	system with	and germination rate,	2022)
	nitrogen	Improved hydrophilicity and	
		water uptake due to surface	
		modifications	
Mung bean	Plasma jet system	Increased germination rate and	(Le et al., 2022)(Le et
	with argon	radicle length,	al., 2022)
		Improvement in water uptake	
		due to surface modifications	
		and changes in water contact	
		angle	
Barley	Dielectric Barrier	Improved seedling shoot	(Benabderrahim et
(<i>Hordeum</i>	Discharge plasma	growth and increased surface	al.,
<i>vulgare</i> L.)	system with	hydrophilicity	2024)(Benabderrahim
	atmospheric air		et al., 2024)

2.7.2 Microbial inactivation and Shelf-life extension of foods

HVACP is widely used in antimicrobial applications, particularly due to the antimicrobial effects of Reactive Oxygen Species (ROS) and Reactive Nitrogen Species (RNS) produced during plasma

treatment. (Sarangapani et al., 2018)(Sarangapani et al., 2018). Thus, it is applied to inactivate pathogenic bacteria such as *Escherichia coli*, *Staphylococcus aureus* (Han et al., 2016)(Han et al., 2016); *Listeria monocytogenes* (Han, Ziuzina, et al., 2016)(Han, Ziuzina, et al., 2016); *Salmonella enterica* (Xu et al., 2017),(Xu et al., 2017), as well as fungal spores of *Aspergillus flavus* (Ott et al., 2021)(Ott et al., 2021) by different mechanisms such as cell leakage, low-level DNA damage, and intracellular damage (Han et al., 2016)(Han et al., 2016). HVACP also inactivates fungal toxins such as deoxynivalenol toxin (Ott et al., 2021)(Ott et al., 2021), Aflatoxin B₁ (Tang et al., 2024),(Tang et al., 2024), and Aflatoxin M₁ (Nguyen et al., 2022)(Nguyen et al., 2022) (**Table 4**). Thus, HVACP has many applications for improving food safety in items such as meat, dairy, and cereal-based products.

In addition to microbial reduction, cold plasma treatment can delay spoilage processes by reducing surface contamination and inhibiting enzymatic activity, thereby extending the shelf life of perishable foods. Studies that focus on impacting the endogenous enzymes are mainly associated with numerous enzymes such as pectin methylesterase (PME), polyphenol oxidase (PPO), peroxidase (POD), superoxide dismutase (SOD), lysozyme, α -chymotrypsin, alkaline phosphatase, catalase (CAT), and lipase (Han et al., 2019)(Han et al., 2019). Importantly, because cold plasma operates at ambient or near-ambient temperatures, it minimizes adverse effects on sensory attributes, nutritional quality, and overall food integrity compared to conventional thermal treatments (Pan et al., 2019)(Pan et al., 2019). Furthermore, cold plasma is a chemical-free and environmentally friendly technology, as reactive species rapidly revert to stable molecules after treatment, leaving no harmful residues. These advantages make cold plasma a promising alternative for enhancing food safety and extending shelf life while meeting the growing demand for minimally processed foods.

Table 4: Effect of cold plasma treatment on food-borne pathogens on food items

Microorg anism	Food substance	Cold Treatment	Plasma	Effects Observed	Reference
<i>E. coli</i>	Lettuce,	Plasma jet system (3.95		The highest inactivation (1.6	(Bermúdez-
	carrots,	kV upto 12.83 kV at 60		log) was recorded at the	Aguirre et
	tomatoes	Hz; 30 sec - 10 min; Argon; flow rate 455.33 sccm)		highest voltage level. More inactivation was recorded in lettuce (1.6 log) followed by tomato (1.4 log).	al., 2013)(Berm údez- Aguirre et al., 2013)
	Almonds	Plasma jet system (549 W at 47 kHz; 10 sec and 20 sec; dry air and nitrogen; flow rate 60 psi: Distance from emitter - 2, 4, or 6 cm)		Shorter treatments recorded better reductions ($N_0 - N \sim -$ 1.00 log CFU/ml) compared to longer treatments ($N_0 - N$ $\sim - 0.70$ log CFU/ml) Nitrogen had a better inactivation effect ($N_0 - N \sim$ $- 0.90$ log CFU/ml) compared to air ($N_0 - N \sim -$ 0.50 log CFU/ml)	(Niemira, 2012)(Niem ira, 2012)
	Cherry tomatoes, strawberries	Dielectric barrier discharge system (70 kVRMS; 30 - 300 sec;		Longer treatment times were needed to reduce <i>Salmonella</i> , <i>E. coli</i> and <i>L. m</i>	(Ziuzina et al., 2014)(Ziuzi

		atmospheric air; Distance from emitter – 140-160 mm)	<i>onocytogenes</i> bacterial populations by 3.5, 3.8 and 4.2 log ₁₀ CFU/sample in strawberries than in tomatoes, due to structural features.	na et al., 2014)
	Apples	Gliding arc plasma system (15 kV at 60 Hz; 1 min and 2 min; dry air; flow rate 20 L/min and 40 L/min: Distance from emitter – 8 mm)	A higher gas flow rate was more effective in microbial inactivation (3.4 to 3.6 log CFU/ml) compared to lower flow rates (2.6 to 3 log CFU/ml).	(Niemira & Sites, 2008)(Niem ira & Sites, 2008)
<i>Salmonell</i> <i>a sp.</i>	Cherry tomatoes	Microwave-powered cold plasma treatment (400–900 W; 2– 10 min; helium (He) and He-oxygen (O ₂) gas mixture)	He-oxygen mixture achieved microbial reduction (3.5 log CFU/tomato) at a lower power and time requirement	(Kim & Min, 2017)(Kim & Min, 2017)
	In-shell pecans, cherry tomatoes	Dielectric barrier discharge system (7 kV; 2,4 min; atmospheric air;	Microbial inactivation efficiency decreased (~3.5 to ~2 log CFU/mL) with	(Timmons et al., 2018)(Timm

	Distance from emitter – 1,3,5,7 cm)	increasing distance and shorter treatment times.	ons et al., 2018)
Unpeeled almonds	Diffuse coplanar surface barrier discharge system (20 kV at 15 Hz; 0-15 min; dry air, O ₂ , N ₂ , CO ₂ , and a 90% CO ₂ + 10% Ar mixture)	Inactivation depends on the gas used, with the highest inactivation occurring with atmospheric air (> 5.0 log ₁₀), followed by O ₂ (4.8 log ₁₀) and N ₂ (2.0 log ₁₀).	(Hertwig et al., 2017)(Hert wig et al., 2017)
Shell eggs	Dielectric barrier discharge system (80 kV and 100 kVRMS at 60 Hz; 0.5 - 15 min; air (21% O ₂ , 78% N ₂ , 1% other gases) and modified air MA65 (65% O ₂ , 30% CO ₂ and 5% N ₂)	Higher voltage and short treatment times resulted in optimal reduction.	(Illera et al., 2022)(Illera et al., 2022)
Radish sprouts	Microwave-powered cold plasma treatment (900 W, nitrogen;	Highest reduction (2.6 log CFU/g) recorded at the longest treatment time,	(Oh et al., 2017)(Oh et al., 2017)

		667 Pa; 0, 2, 5, 10, and 20 min)	with no significant effect on quality characteristics.	
<i>Listeria monocytogenes</i>	Ready-to-eat ham	Dielectric barrier discharge system (0–28 kV; 30-180 sec; atmospheric air)	The greatest reduction was observed at the longest treatment time. Treatment temperature did not affect the reduction levels.	(Yadav et al., 2019)(Yadav et al., 2019)
	In-shell pecans, cherry tomatoes	Dielectric barrier discharge system (7 kV; 2,4 min; atmospheric air; Distance from emitter – 1,3,5,7 cm)	Microbial inactivation efficiency decreased with increasing distance and shorter treatment times.	(Timmons et al., 2018)(Timmons et al., 2018)
	Romaine lettuce	Dielectric barrier discharge system (34.8 kV; 5 min; atmospheric air, 5% O ₂ + 95% N ₂ , 10% O ₂ + 90% N ₂ gas mixtures; in-package treatment)	Microbial inactivation is not affected by package type or moisture content.	(Min et al., 2016)(Min et al., 2016)
	Tender coconut water	Dielectric barrier discharge system (90	Better microbial reduction with modified air M65 as	(Mahnot et al.,

		kV; 120 sec; dry air (78% N ₂ , 21% O ₂ and traces of other gases) and modified air M65 (65% O ₂ , 30% CO ₂ , 5% N ₂); 0.6 L/min)	indicated by significantly lower Total Plate Count and Total Coliform value	2019)(Mahn ot et al., 2019)
<i>Staphylococcus aureus</i>	Dry-cured beef	Jet plasma system (25 kV at 42 kHz; 180 and 300 sec; argon, oxygen, and mixture of both)	Increased inactivation (0.85 log CFU/cm ²) at longer treatment times and with oxygen gas.	(Gök et al., 2019)(Gök et al., 2019)
	Strawberries	Jet plasma system (5, 10, 15 min; (98% Ar and 2% O ₂ mixture; 5 L/min)	Microbial reduction (1.6 to 2.3 log) achieved with the plasma-activated water (PAW) Reduction efficiency depended on the treatment time for PAW.	(Ma et al., 2015)(Ma et al., 2015)
	Dried blackmouth angler	Dielectric barrier discharge system (3 kV at 43 kHz; 0, 1, 5, 10, 20, and 30 min; nitrogen; 1.5 L/min;	The highest reduction (1.03 log ₁₀ CFU/g) occurred at the longest treatment duration, with a slight decrease in the quality characteristics.	(Choi et al., 2020)(Choi et al., 2020)

Distance from emitter

– 3 mm)

2.7.3 Plasma effects on nutritional and functional quality

The potential of HVACP extends beyond food-safety applications to enhancing the nutritional and functional properties of proteins through modification. HVACP causes protein modification through several mechanisms, including protein-protein aggregation, protein unfolding and secondary and tertiary structural alterations, peptide bond cleavage, and amino acid modification. Reactive species generated during plasma treatment interact with proteins, denaturing them, altering their tertiary structure, and inducing disulfide cross-linking. The unfolding of protein molecules led to protein-protein interactions and protein aggregation (Xu et al., 2021)(Xu et al., 2021). Protein modification can result from direct interaction with reactive species or indirect interaction with secondary oxidation products (Olatunde et al., 2023)(Olatunde et al., 2023). HVACP can also alter sulfur-containing amino acids and techno-functional properties, including structure, enzymatic activity, and solubility (Olatunde et al., 2021)(Olatunde et al., 2021).

Functional properties are associated with the physicochemical behaviors of food constituents that determine their performance during processing, formulation, and consumption, and they are particularly critical for proteins used as food ingredients. Protein solubility is considered one of the most important functional properties, as it directly influences other properties such as emulsification, foaming, and gelation (Cheng et al., 2023)(Cheng et al., 2023). Cold plasma treatment has been shown to influence protein solubility through plasma-induced conformational changes, including partial unfolding, disruption of non-covalent interactions, and oxidation of specific amino acid residues. These effects increase the exposure of hydrophilic groups and

enhance protein–water interactions under optimized conditions, while excessive treatment can promote aggregation and reduce solubility (Deng et al., 2025)(Deng et al., 2025).

Emulsifying properties reflect a protein’s ability to adsorb at oil–water interfaces, reduce interfacial tension, and stabilize dispersed lipid droplets. Cold plasma treatment can improve emulsifying activity and stability by increasing surface hydrophobicity and molecular flexibility, allowing proteins to more rapidly orient and anchor at interfaces. Plasma-induced exposure of both hydrophilic and hydrophobic domains enhances interfacial film formation, although over-oxidation may weaken interfacial integrity (Zhang et al., 2022)(Zhang et al., 2022). Foaming properties, including foam capacity and stability, focus on the ability of proteins to entrap air and form viscoelastic films around gas bubbles (Hosseini et al., 2025)(Hosseini et al., 2025). Cold plasma treatment may enhance foaming performance by increasing protein flexibility and interfacial adsorption kinetics, enabling more stable air–water interfaces. However, excessive oxidation can lead to rigid protein structures that limit foam formation (Rout & Srivastav, 2024)(Rout & Srivastav, 2024). Water- and oil-holding capacities largely contribute to the texture and mouthfeel of food products. Plasma treatment can enhance these properties by increasing surface area, porosity, and the availability of polar and non-polar binding sites, thereby improving interactions with water and lipids (Zare et al., 2022)(Zare et al., 2022). Gelation properties describe the ability of proteins to form three-dimensional networks upon heating or enzymatic action. Cold plasma can alter gel strength and network structure by modifying protein–protein interactions, often promoting more uniform and cohesive gels under controlled treatment conditions (Eazhumalai et al., 2025)(Eazhumalai et al., 2025). Overall, HVACP has high potential to enhance protein functionality and address key drawbacks of protein concentrates, thereby further expanding their utility across various food systems.

2.8 Conclusion

Overall, the literature review presents a collection of insightful information which bears great importance given the growing demand for sustainable protein sources and the increasing application of plant-based protein ingredients in meeting global nutritional needs. Introduction of sustainability aspects into the food system has diverted the attention towards dry fractionation, particularly air classification, as a promising environmentally friendly approach for producing protein concentrates. However, its efficiency is influenced by several operational and material-related factors, necessitating optimization of the processing parameters. Despite these advancements, plant protein concentrates often exhibit limited techno-functional properties, which restrict their broader application in food systems. Consequently, various modification approaches, including physical, chemical, and biological methods, have been explored to enhance functionality. Among them, high-voltage atmospheric cold plasma (HVACP), has gained attention as an innovative non-thermal technique capable of modifying protein structure and improving functional properties with minimal effect to the native protein structure and nutritional quality. Its applications in agriculture and food processing further demonstrate its potential for improving microbial safety, shelf-life, and overall product quality. Therefore, integrating sustainable fractionation techniques with emerging modification technologies such as HVACP presents a promising strategy for developing high-quality plant protein ingredients, forming the basis for the present study.

Author contributions

Shimani Attygalle: Conceptualization, literature collection, writing-original draft, writing-review and editing

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Chapter 3: Optimizing Air Classification Conditions for Lentil Protein Concentrates for Functionality Improvement

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Abstract

Air classification is a sustainable, less resource-intensive process for producing plant protein ingredients. This study explored the effects of dehulling and air classifier wheel speed on the compositional, structural, thermal, and techno-functional properties of green lentil protein concentrates. Whole (NDH) and dehulled (DH) lentil seeds were processed using a pilot-scale air classifier at 30 and 40 Hz. Proximate composition, particle size, amino acid profile, secondary structure, thermal behavior, functional properties, antinutritional factors, and in vitro protein digestibility were analyzed. A two-way ANOVA assessed the effects of dehulling and wheel speed ($p < 0.05$). Air classification performance parameters remained unaffected by processing variables. Protein (51.18–54.29%) and crude fat contents (2.03–2.35%) increased at 40 Hz. DH samples exhibited the smallest particle sizes. Several amino acids, including tyrosine, tryptophan, histidine, proline, and aspartic acid, were more abundant at 40 Hz. DH samples showed higher onset and peak denaturation temperatures but lower enthalpy. β -sheet structures predominated in NDH samples at 30 Hz, while β -turns were more prominent in DH samples. Functional properties were significantly influenced by both variables, affecting protein solubility, emulsifying, foaming, and

gelling behaviors. DH samples at 40 Hz achieved the lowest gelation concentration (4%). Total phenolic content, phytic acid content, and in vitro protein digestibility were also significantly affected. Overall, dehulling and wheel speed are crucial parameters for tailoring the functionality of lentil protein concentrate.

Keywords – Air classification, protein concentrates, processing conditions, optimization

3.1 Introduction

Plant protein ingredients are gaining popularity in food formulation due to their numerous advantages for food products and the industry. Protein isolates and concentrates differ in their protein contents and related properties (Meganaharshini et al., 2023)(Meganaharshini et al., 2023). According to the Codex Alimentarius Commission, the protein contents of protein concentrates and isolates are 65-90% and above 90%, respectively (Codex, 2022)(Codex, 2022). Two extraction techniques, namely dry (DF) and wet (WE), are used to produce protein concentrates and isolates, respectively. Despite their ability to produce highly pure protein isolates, wet extraction methods have the disadvantages of high water and energy requirements and adverse effects on protein functionality. Wet extraction methods usually employ harsh temperature and pH conditions, which cause a loss of protein functionality (Pelgrom, Boom, et al., 2015)(Pelgrom, Boom, et al., 2015). In contrast, dry fractionation has several advantages over wet extraction, particularly in terms of industrial feasibility and sustainability. The energy requirement for the dry fractionation process is lower than that for wet fractionation (Schutyser & van der Goot, 2011), as it avoids adding chemicals or large amounts of water, leading to lower wastewater generation and fewer energy-consuming drying steps (Fernando, 2021)(Fernando, 2021). The drawbacks of dry fractionation include lower protein content and other impurities, which can negatively affect the storage and processing of protein ingredients (De Angelis et al., 2024)(De Angelis et al., 2024).

Protein concentrates are primarily produced by dry fractionation (DF), which often involves milling and air classification. The density and size of particles determine their aerodynamic behavior, which in turn determines the air classification efficiency of the process (Schutyser & van der Goot, 2011)(Schutyser & van der Goot, 2011). Upon air classification, the material is separated into fine and coarse fractions based on the cut size set for the process, allowing particles such as

starch granules with a size above the cut size move into the coarse fraction, while smaller particles, such as protein particles, move into the fine fraction (Schutyser & van der Goot, 2011)(Schutyser & van der Goot, 2011). Dry fractionation can be improved by employing various strategies, including milling the finer fraction, adjusting air classifier wheel parameters, plant breeding to modify grain characteristics, and grain pretreatments such as dehulling (Pelgrom, Boom, et al., 2015)(Pelgrom, Boom, et al., 2015). Improved air classification efficiency can thus be achieved by systematically evaluating and optimizing the factors that influence the process.

There are several factors affecting the efficiency of air classification. Air classifier wheel speed is the factor most extensively studied in relation to air classification efficiency. Several studies have been conducted to assess the impact of air classifier wheel speed on the protein separation in different types of flour, mung bean, yellow pea, and cowpea (Schlangen et al., 2022)(Schlangen et al., 2022); field peas (Wang & Maximiuk, 2019);(Wang & Maximiuk, 2019); (Pelgrom et al., 2013)(Pelgrom et al., 2013); fava bean (Oluwajuyitan & Aluko, 2025)(Oluwajuyitan & Aluko, 2025); barley (Silventoinen et al., 2018)(Silventoinen et al., 2018); and brown lentils (Funke, Loeffler, et al., 2022)(Funke, Loeffler, et al., 2022). The protein content in the fine fraction increased with increasing classifier wheel speed. Dehulling is a standard pretreatment applied before air classification and involves removing the seed coat (hull) from the seeds. The degree of hull adhesion may vary with the type of pulse; consequently, it is associated with different levels of difficulty in removing the hull. Tightly adhering seed coats are removed by abrasive-type dehullers, whereas loose hulls are removed by attrition-type dehullers like hammer mills (Fernando, 2021a)(Fernando, 2021a). Dehulling has been reported to positively affect protein separation efficiency by influencing material flow properties (Saldanha do Carmo et al., 2020).

Few studies have investigated the effect of dehulling on the efficiency of the dry fractionation process, underscoring the need for further research in this area.

Thus, a significant knowledge gap exists on the influence of dry fractionation process parameters on the structural and functional characteristics of protein concentrates. Funke, Loeffler, et al. (2022)Funke, Loeffler, et al. (2022) explored the properties of lentil protein concentrates, yet the main focus was limited to understanding the interfacial properties and emulsifying behaviors of these protein ingredients. Therefore, the impact of air classification on other techno-functional properties, secondary structure, thermal properties, phytochemical composition, and digestibility of lentil protein concentrates remains largely underexplored. Therefore, to address this gap, the present study tested the hypothesis that dehulling and air classifier wheel speed significantly affect the composition and techno-functional properties of lentil protein concentrates. The objective of the study is to determine the optimum combination of dehulling and air classifier wheel speed to produce lentil protein concentrates with higher protein content and improved chemical, structural and techno-functional properties.

3.2 Materials and Methods

3.2.1 Air classification and performance parameters

3.2.1.1 Air classification

Laird green lentils (*Lens culinaris*) were purchased from AGT Foods (Regina, SK, Canada). The whole batch of purchased seeds was milled using a rotor beater mill (SR 300, Retsch GmbH, Germany). Half of the batch (125 kg) was dehulled using Rice Whitener (RMB 10G, SATAKE Group, USA), and the remaining half was used as whole seeds. This variable was identified as the ‘type of seed’ (whole (NDH); dehulled (DH)). Each batch was then subjected to air classification using a Prater-Sterling air classifier (MAC-0, Prater, USA) at two wheel speeds: 30 Hz (4292 rpm)

and 40 Hz (5723 rpm). Each round of air classification yielded a fine fraction and a coarse fraction. Each fraction was obtained in duplicates through two fractionation trials for each combination of ‘type of seed’ and ‘air classifier wheel speed’. Random samples were collected from these batches and fractions for further analysis and stored at 4 °C. Based on the mass of yielded fractions, several parameters related to air classification performance were calculated.

3.3.1.2 Protein Yield

Protein yield is defined as the mass percentage of the original flour recovered into the protein fraction (the fine fraction) (Schutyser & van der Goot, 2011). It was calculated using the formula given below.

$$\text{Mass Yield \%} \left(\frac{w}{w} \right) = \frac{\text{Dry weight of fine fraction (kg)}}{\text{Dry weight of feed (kg)}}$$

3.3.1.3 Protein Separation Efficiency (PSE%)

Protein separation efficiency (PSE) is the percentage of the total flour protein recovered in the protein fraction (Schutyser & van der Goot, 2011)(Schutyser & van der Goot, 2011). It was calculated using the formula given below.

$$\text{PSE \%} = \frac{\text{Dry weight of fraction (kg)} \times \text{Protein content of fraction (\% db)}}{\text{Dry weight of feed (kg)} \times \text{Protein content of feed (\% db)}}$$

3.3.1.4 Protein Shift Percentage (δ)

Protein shift percentage is the percentage of protein that is shifted from the original flour into the protein fraction (Schutyser & van der Goot, 2011)(Schutyser & van der Goot, 2011). It was calculated using the formula given below.

$$\delta = \frac{1}{P_{flour}} [(P_P - P_{flour}) \cdot \Phi_P]$$

where, Φ_P , P_{flour} , and P_P are the protein fraction yield and the protein percentage of the flour, and the protein fraction.

3.2.2 Alkaline Extraction of Protein Isolate

Protein isolate (PI) extracted through conventional alkaline extraction was used as a positive control in the study. Alkaline extraction was performed according to the method described by Karaca et al. (2011) Karaca et al. (2011) with some modifications. Lentil flour (<0.2 mm) was dispersed in Milli-Q water in a 1:10 (w/v) ratio and thoroughly mixed for 10-15 sec. Following homogenization (Model 850, Fisher Scientific, Ottawa, ON, Canada) at 14,000 rpm for 1 min, the solution was adjusted to pH 9 with 5 M NaOH, and protein was extracted (250 rpm, 2 hrs, room temperature). The obtained alkali extract was centrifuged (12,100 g, 30 min, 4 °C), and the supernatant pH was adjusted to 4.5 with 2.5 M HCl. Precipitation was allowed to proceed overnight at 4 °C, and the precipitates were collected. Pellets were then washed twice with the same centrifugation step as above. Pellets were then redispersed in water, the pH adjusted to 7, and freeze-dried at -21 °C (HRFDXL, Harvest Right™, USA). The resulting isolates were stored at -20 °C. Accordingly, 4 fine fractions (protein-rich fractions/ concentrates), whole seed flour (F) (negative control), and alkaline extracted protein isolate (positive control) were used for the analysis.

3.2.3 Proximate Analysis

The moisture content of the samples was analyzed by drying at 105 °C (Isotemp 60L Oven Gravity, Thermo Electron, USA) for 18 hrs until a constant weight was achieved. Protein content was measured by combustion analysis, as outlined in AACC International Method 46-30.01 (AACC International, 2011). A nitrogen and protein analyzer (Rapid N exceed®, Elementar, Germany),

calibrated with aspartic acid (10.42 g N/100 g), was used for the analysis. The conversion factor 5.7 is used to convert nitrogen (% w/w) to crude protein (% w/w) content (Shen et al., 2024)(Shen et al., 2024). The ash content was determined by placing the samples in a muffle furnace (Model 1100 box furnace, Thermo Scientific, USA) at 550 °C overnight. Crude fat content was determined using a fat extractor (E-500, BUCHI Labortechnik, Switzerland). The Megazyme enzyme assay kit (K-TSTA-100A, Megazyme International, Ireland) was used to determine total starch (AOAC 996.11). All analyses were conducted in triplicate.

3.2.4 Particle size determination

Particle size distribution of the samples was determined using a dynamic light scattering instrument (Mastersizer 3000, Malvern Panalytical Ltd., UK) equipped with a powder dispersion unit (Aero S feeder, Malvern Panalytical Ltd., UK). Dry powder was placed on the vibrating tray, and uniform dispersion was achieved by vibrating the samples into the analyzer. Data acquisition and processing were carried out using Mastersizer software version 3.8.5. Results were reported as cumulative particle size percentiles (D_{10} , D_{50} , and D_{90}), representing the particle diameters below which 10%, 50%, and 90% of the total sample volume are distributed, respectively.

3.2.5 Amino acid analysis

Modified versions of the methods AOAC 982.30, AOAC 994.12, and ISO 13904:2005(E) were used to determine the regular amino acid, oxidized amino acid, and Tryptophan content, respectively. Quantification of most amino acids was performed based on AOAC Official Method 982.30 (AOAC, 2012)(AOAC, 2012), by subjecting to 24 hrs acid hydrolysis (HCl, 6N), followed by the analysis. Methionine and Cysteine were determined based on AOAC Official Method 985.28. Initially, samples were oxidized with performic acid and subsequently subjected to

hydrolysis and analysis. Tryptophan was analyzed separately following ISO 13904:2016 (ISO, 2016)(ISO, 2016). The hydrolysates were derivatized and separated using a UPLC system (Kyoto, Japan) equipped with a Waters AccQ C18 column (100 mm × 2.1 mm, 1.7 μm). Amino acid concentrations were calculated based on hydrated molecular weights, and values reported as % (w/w) were converted to mg/g protein.

3.2.6 Changes in protein secondary structures

Fourier transform infrared spectra were collected using an attenuated total reflectance (ATR) module attached to an FTIR spectrophotometer (INVENIO S, Bruker Scientific LLC, USA) and OPUS software. The acquisition conditions were set to the 400-4000⁻¹ spectral range, 4 cm⁻¹ resolution, and 120 scans per sample. For each sample, three spectra were collected at room temperature, with a new background recorded (120 scans) before each sample measurement. Once the powdered sample was placed under the ATR diamond crystal, the same pressure was applied to all samples to ensure uniformity. Data analysis was performed using OriginPro 2025 (OriginLab Corporation, USA) in the Amide I region (1600-1700 cm⁻¹). Following background subtraction, the selected area was normalized (0-1), and a second derivative was created using a Savitzky-Golay second-order function. The secondary structures were identified by peak fitting of the second derivative.

3.2.7 SDS-PAGE analysis

SDS-PAGE analysis is used to characterize the polypeptide composition of the samples, as described by Laemmli (1970) with modifications (Raikos et al., 2014), using a Mini-Protein electrophoresis unit (Bio-Rad Laboratories Inc., USA) with slight modifications. Protein dispersions of 4 mg/ml (w/v) concentration were prepared using 5% sodium dodecyl sulfate

solution, by heating (85 °C, 1 hrs), followed by centrifugation (12,310 g, 10 min). Final samples of protein concentration of 2 mg/mL (v/v) were prepared by mixing equal volumes of Laemmli buffer and protein sample, while samples under reducing conditions were added with 2-mercaptoethanol. All samples were then heated (90 °C, 5 min). Aliquots of 5 µL of each prepared sample and 10 µL of Precision Plus Protein Standard were loaded onto a 4–15% Mini-PROTEAN® TGX™ precast gel. Electrophoresis was carried out at 150 V for 1 hrs using a Mini-PROTEAN II cell. Gels were then stained with Coomassie Brilliant Blue R-250 for 1 hrs and destained for 2 hrs using a solution of 50% methanol and 10% acetic acid in water. Protein bands were visualized using the Bio-Rad Imaging System (ChemiDoc™, Bio-Rad Laboratories Inc., USA).

3.2.8 Morphological Characteristics

Scanning electron microscopy (SEM) (Quanta 650 FEG, FEI Company, USA) equipped with an Everhart-Thornley (ET) detector was used to examine the morphological features of the samples. Samples were mounted onto aluminum SEM stubs using double-sided conductive carbon tape and spread as a thin layer prior to imaging. Then samples were coated with a gold-palladium (Au–Pd) layer using a sputter coater (Desk II, Denton Vacuum Inc., USA) operated at 50 mTorr pressure, 45 mA current, and a coating time of 60 seconds. The coated samples were then dried under nitrogen purging at a vacuum in the same instrument. Microscopic images were acquired and analyzed using xT Microscope Control software (version 6.2.8, FEI Company, USA) at magnification powers x500, x1000, and x5000.

3.2.9 Techno-functional Properties

3.2.9.1 Water Holding Capacity (WHC)

The method specified by Malomo et al. (2014) was used to determine the samples' water-holding capacity with modifications. Analysis was conducted in triplicate. Samples of 0.15 g were measured into pre-weighed Eppendorf tubes, added with 1.5 mL of distilled water, vortexed (2 min), and kept standing for 30 min, allowing the sample to hold water. Samples were then centrifuged (7000 g, 25 min, at room temperature), and supernatants were discarded. Excess water in the tubes was drained, and the weight of the remaining sample and water in the tube was measured after 15 min. The WHC of each sample is calculated using the equation below.

$$WHC \% = \frac{W2 - (W1 + \text{Sample weight})}{\text{Sample weight}} \times 100$$

Where, W1-empty weight of Eppendorf tube; W2-weight of tube + sample + retained water

3.2.9.2 Oil Holding Capacity (OHC)

Same procedure outlined for WHC determination as mentioned in section 3.3.10.1 was followed to determine the oil-holding capacity of the samples, with distilled water replaced with canola oil (Malomo et al., 2014)(Malomo et al., 2014). It was calculated using the equation below.

$$OHC \% = \frac{W2 - (W1 + \text{Sample weight})}{\text{Sample weight}} \times 100$$

Where, W1-empty weight of Eppendorf tube; W2-weight of tube + sample + retained oil.

3.2.9.3 Protein Solubility

The protein solubility of the concentrates in pH 3, 5, 7, and 9 was determined following the method indicated by Barac et al. (2010), with slight modifications. Protein dispersions at 0.5% (w/v) in Milli-Q water were stirred for 10 min and then hydrated overnight at 4 °C. These samples were

stirred again for 1 hrs, pH adjusted by adding 0.1 M HCl or 0.1 M NaOH, and stirred again for 1 hrs, to facilitate the protein solubility at the respective pH. Supernatants obtained by centrifugation (15,000 g, 15 min, 22 °C) were measured using the measuring cylinder and stored at 4 °C for future analysis. Protein content in the supernatant was quantified using the Bradford method. Protein solubility is determined the following equation.

$$\text{Protein solubility \%} = \frac{\text{Soluble protein content}}{\text{Protein content in the initial sample}} \times 100$$

3.2.9.4 Emulsifying Activity Index (EAI) and Emulsion Stability Index (ESI)

The analyses were conducted using the method described by Karaca et al. (2011), with minor modifications. The following 0.1 M buffer systems were used: glycine–HCl (pH 3), sodium acetate (pH 5), sodium phosphate (pH 7), and Tris (pH 9). Protein dispersions of 1% were prepared and added with 5 mL of canola oil, followed by vortexing (15 s) and homogenization (2000 rpm, 1 min) (Model 850, Fisher Scientific, Canada). An aliquot of 50 µL of emulsion was immediately collected from the bottom of the tube and diluted with 5 mL of 0.1% sodium dodecyl sulphate and vortexed for 10 s to ensure proper mixing. The diluted samples were then measured for absorbance at 500 nm using a UV-Visible Spectrophotometer (GENESYS 150, Thermo Scientific, USA). After 10 min, another aliquot of 50 µL is taken from the bottom of the emulsion, and the same procedure is followed to measure the absorbance after the dilution. The equations below were used to calculate EAI and ESI. .

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

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

Where A₀ - absorbance of the diluted emulsion at 0 min; ΔA - differences between the absorbances of the diluted emulsion at 0 min and 10 min; N - dilution factor of the emulsion; C - Protein concentration in g/ml in aqueous phase before emulsion formation; φ - Volume fraction of the oil in total emulsion; t - time interval of emulsion storage.

3.2.9.5 Foaming Capacity (FC) and Foaming Stability (FS)

The method described in Ajibola & Aluko (2022), with some modifications, Ajibola & Aluko (2022), with some modifications, was used in the analysis. The 0.1 M buffer systems, namely, glycine–HCl (pH 3), sodium acetate (pH 5), sodium phosphate (pH 7), and Tris (pH 9), were used in the analysis. Protein dispersions of 1% were prepared and homogenized (17000 rpm, 2 min) (Model 850, Fisher Scientific, Canada). Immediately after homogenization, the foam height was measured with a ruler (at 0 min). Dispersions were then left aside for 30 min, and the height of the remaining foam was measured. The foam volume was determined by converting the height values to volume using the tube measurements. FC and FS were calculated according to the following equations. .

$$\text{Foaming capacity} = \frac{V_0}{V} \times 100$$

$$\text{Foaming stability} = \frac{V_{30}}{V_0} \times 100$$

Where, V - volume of the protein dispersion before foaming; V₀ - volume of the foam generated at 0 min; V₃₀ - volume of the remaining foam at 30 min

3.2.10 Phytochemical composition

Total phenolic content was determined by following the Folin-Ciocalteu reagent method (Velioglu et al., 1998)(Velioglu et al., 1998) with modifications. Extraction was performed using 2 mL of

80% methanol (1% HCl) for 200 mg of each sample, incubated for 2 hrs at room temperature. Solutions were then centrifuged (2000 g, 15 min), and an aliquot of 200 µL of the supernatant was mixed with 1.5 mL of 10-fold diluted Folin-Ciocalteu reagent, kept in the dark (22 °C, 5 min). Solutions were then incubated in the dark (22 °C, 90 min) after the addition of 1.5 mL Sodium bicarbonate (60 g/L). Absorbance was measured at 725 nm using a UV-Visible Spectrophotometer (GENESYS 150, Thermo Scientific, USA) against the gallic acid standard curve. Results were calculated in gallic acid equivalents. Phytic acid content was determined using the Megazyme Phytic acid (phytate)/ Total Phosphorus assay kit (K-PHYT, Megazyme International, Ireland). Analyses were conducted in triplicate.

3.3.11 In Vitro Protein Digestibility and PDCAAS Calculation

In Vitro Protein Digestibility (IVPD) values of the samples were determined by using the pH-drop method (AOCS, 2024; Goldberg et al., 2026). Sample solutions containing 1 mg N/mL were prepared, the pH of each solution was adjusted to 8, and the solutions were incubated at 37 °C. Enzymatic digestion was performed using a mixture of proteolytic enzymes containing trypsin (1.6 mg/mL), α -chymotrypsin (3.1 mg/mL), and *Streptomyces griseus* peptidase (1.3 mg/mL), added to the protein solution at a 1:10 (v/v) ratio while stirring continuously at 37 °C. The pH change after 10 min (Δ pH) was recorded, and the IVPD was calculated as follows.

$$\text{IVPD} = [74.51 + (22.51 * \Delta\text{pH})]$$

The PDCAAS values were calculated in accordance with the method outlined by FAO/WHO (1991). The content of each amino acid in the samples was determined as described in Section 2.4 and expressed as mg/g protein. The amino acid score (AAS) was obtained by dividing the content of each indispensable amino acid in the test protein by the corresponding requirement specified in

the FAO/WHO reference pattern for preschool children. The lowest ratio among the indispensable amino acids was identified as the limiting amino acid score.

The PDCAAS was then calculated by multiplying the amino acid score of the limiting amino acid by the corresponding protein digestibility value, using the equation provided below.

$$PDCAAS = \text{Amino Acid Score} \times \text{Protein Digestibility}$$

3.3.12 Statistical Analysis

Data are expressed as the means of replicates. To assess the impact of the variables, two-way analysis of variance ANOVA followed by Tukey's HSD test for multiple comparisons is used. To compare the concentrates with the negative and positive controls (flour and protein isolate, respectively), one-way ANOVA followed by Tukey's HSD test for multiple comparisons is used. The significance level was set at $p < 0.05$. Statistical analyses are conducted using Minitab 22 Statistical Software (Minitab Inc., State College, PA, USA).

3.3 Results and Discussion

3.3.1 Air Classification Optimization

The air classification process involves monitoring various parameters to optimize its efficiency. The present study results do not indicate a significant impact of dehulling or air classifier wheel speed on the three parameters considered: protein yield, protein separation efficiency (PSE), and protein shift percentage. Lack of interaction between processing parameters and lentil dry fractionation could be due to an imbalance in the operating rotational speed and air flow rate, which can affect the smooth particle motion between the blades (Abohelwa et al., 2023)(Abohelwa et al., 2023). However, many studies have reported conflicting findings, particularly regarding the effect of wheel speed. A decline in protein yield with increasing protein content is commonly

observed. This trend was evident in the work of Schlangen et al. (2022), who studied mung bean, cowpea, and yellow pea, and in the findings of Pulivarthi et al. (2025) for red lentils. In barley, Silventoinen et al. (2018) also reported a decrease in protein yield; however, the reduction was less pronounced than in other studies, suggesting a more efficient protein separation process. Similarly, Wang & Maximiuk (2019) observed a decrease in fine fraction yield and an increase in coarse-fraction yield for field peas as the classifier wheel speed increased from 2,500 to 4,500 rpm. Interestingly, this trend reversed when the wheel speed exceeded 4,500 rpm. Oluwajuyitan & Aluko (2025) also reported comparable results for fava bean flour: the coarse fraction yield increased up to a wheel speed of 30 Hz but declined beyond 35 Hz, accompanied by reduced separation efficiency and a corresponding increase in fine fraction yield. The study's range of wheel speeds was insufficient to produce a significant difference, highlighting the need for further research.

Several studies have examined the effects of dehulling on these parameters (Saldanha do Carmo et al., 2022; Singh et al., 2024). In peas, protein separation efficiency (PSE) was reported to improve after dehulling. This improvement has been attributed to the possible adherence of protein particles to hull fragments or to the influence of hulls on material flow behavior during air classification. However, this phenomenon was not observed in faba beans, suggesting that the impact of dehulling is material-dependent (Saldanha do Carmo et al., 2020). Although Singh et al. (2024) reported a significantly higher protein content in a hull-less barley variety, they did not associate this increase with the absence of hulls. As observed with faba beans, the protein separation efficiency (PSE) of the dehulled fractions in the present study was generally lower, suggesting that hulls may positively influence material flow during air classification.

3.3.2 Proximate Composition

Table 3.1: Proximate Composition and starch content of fine fractions compared with controls

Sample	Crude Protein %	Crude Fat %	Crude Ash %	Starch %
Whole flour	25.13±0.01 ^d	1.18±0.03 ^c	3.21±0.07 ^c	56.94±1.41 ^a
NDH-30-F	51.34±0.55 ^{c,B}	2.03±0.02 ^{b,B}	5.60±0.02 ^{a,A}	21.27±0.81 ^{b,A}
NDH-40-F	53.30±0.61 ^{bc,AB}	2.18±0.05 ^{ab, AB}	5.55±0.21 ^{ab,A}	17.08±0.18 ^{c,B}
DH-30-F	51.19±0.44 ^{c,B}	2.16±0.07 ^{ab, AB}	5.17±0.10 ^{b,A}	17.77±0.63 ^{c,B}
DH-40-F	54.30±0.46 ^{b,A}	2.35±0.07 ^{a,A}	5.58±0.02 ^{a,A}	8.15±0.00 ^{d,C}
Protein Isolate	83.49±0.95 ^a	0.84±0.01 ^d	5.45±0.05 ^{ab}	1.16±0.03 ^e

(Uppercase superscripts – two-way ANOVA (n=2); lowercase superscripts – one-way ANOVA (n=2); NDH-Whole seeds; DH-Dehulled seeds; 30- 30 Hz; 40- 40Hz; F- Fine fraction; C-Coarse fraction)

The protein content of the fine fractions ranged from 51.19% to 54.30% on a dry basis (**Table 3.1**), representing a protein enrichment of 128.2% to 142.1% achieved through the dry fractionation (DF) process. The data indicated that exposure to air classification at a higher wheel speed significantly increased the protein content of the fine fraction, thereby improving protein separation. This correlates with the effect of wheel speed on the air classification parameters, as discussed in section 3.4.1 with relevance to past findings. The values recorded in this study are comparable to those reported in previous studies but appear to be slightly higher when specific methodological factors are taken into account. Fine fraction obtained from air classifying dehulled seeds at 40 Hz (5723 rpm) recorded the highest protein content among the fine fractions. The value was closely aligned with the protein content of a fine fraction obtained at 9000 rpm (Funke, Loeffler, et al., 2022), that of the fine fraction obtained by re-airclassification at 12,000 rpm

(Funke, Boom, et al., 2022), and commercially obtained DF lentil protein ingredients (De Angelis et al., 2024). However, the present study's protein content values for the fine fractions appeared lower than those reported by Pelgrom et al. (2015), who used 10,000 rpm for the air classification process, and by Sosulski & Youngs (1979). Thus, it is evident that the final protein content of the fine fractions is highly dependent on numerous factors, including the protein content of the starting material, lentil variety, and processing conditions (Funke, Loeffler, et al., 2022).

The crude fat content of the samples (**Table 3.1**) also appears to follow a similar trend, with samples with higher protein content showing higher crude fat content. This suggests that protein-lipid interactions within the seed induce the movement of lipids into the fine fraction, along with proteins, during air classification. Fine fractions obtained from dehulled seeds showed significantly higher crude fat content, probably caused by the reduced amounts of fiber in the starting material. The crude fat content of the samples is highly consistent with the value recorded for commercial lentil protein concentrates De Angelis et al. (2024) and Sosulski & Youngs (1979). The crude fat content of the protein isolate was lower, as alkaline extraction is more effective at separating protein. Crude fat values obtained for lentil flour during the study are also highly consistent with the values in the literature (Caldeira et al., 2024; Funke, Boom, et al., 2022). The ash content of the fine fractions was 5.17–5.84% (dw), while the protein isolate also recorded 5.45% (**Table 3.1**), which falls within the same range. The ash contents of the fine fractions were not significantly different from each other. De Angelis et al. (2024) also recorded two significantly different ash content values for two different commercial lentil protein concentrates, as 5.20% and 3.77%, among which one value closely aligns with the present study's values. Ash content values reported by Sosulski & Youngs (1979) and Funke, Boom, et al. (2022) are 5.5% and 4.80%, respectively.

Starch enrichment of the fine fractions followed the opposite trend to protein enrichment (**Table 3.1**), as expected, since the two phenomena are mutually counteractive. Accordingly, the fine fraction obtained from dehulled seeds at a higher air classification wheel speed had the lowest starch content, indicating better protein separation as wheel speed increased. This is consistent with many prior findings, which attribute this phenomenon to the larger granular size of starch relative to smaller protein bodies (Pelgrom et al., 2013). Larger granular size of starch particles was well apparent in the scanning electron micrographs which are discussed in Section 3.4.3.2. A significant effect of dehulling on the starch content was also observed, as the starch content in both the fine and coarse fractions obtained from dehulled seeds was lower. This is likely due to the lower starch content of the starting material, as the hulls are removed. Accordingly, protein enrichment of the fine fraction can be enhanced by dehulling the starting material and employing a higher air classification wheel speed during air classification.

3.3.3 Characterization of the obtained fractions and raw material

3.3.3.1 Particle size analysis

Table 3.2 shows the particle size distribution as affected by milling conditions. The particle size of protein ingredients plays a critical role in determining their techno-functional properties, which, in turn, influence their suitability for various food product formulations. The average particle sizes of fine fractions were significantly reduced as the wheel speed increased. In contrast, the particle size of the coarse fractions was significantly affected by dehulling, with reduced values in dehulled samples.

Table 3.2: Particle size of fine and coarse fractions

Sample	Particle size in μm
--------	--------------------------------

	Dv (10)	Dv (50)	Dv (90)
NDH-30-F	3.25±0.01 ^c	8.62±0.03 ^e	18.40±0.00 ^e
NDH-40-F	2.48±0.01 ^d	6.55±0.03 ^f	14.69±0.03 ^e
DH-30-F	2.96±0.03 ^c	8.29±0.01 ^e	18.30±0.02 ^g
DH-40-F	2.47±0.02 ^d	6.47±0.02 ^f	14.30±0.01 ^f
NDH-30-C	14.80±0.14 ^a	27.40±0.14 ^a	118.00±0.00 ^a
NDH-40-C	12.85±0.07 ^b	26.10±0.14 ^c	117.00±0.00 ^b
DH-30-C	14.70±0.00 ^a	26.90±0.14 ^b	70.70±0.14 ^c
DH-40-C	13.20±0.28 ^b	25.10±0.14 ^d	66.85±0.21 ^d

(Uppercase superscripts – two-way ANOVA (n=2); lowercase superscripts – one-way ANOVA (n=2); NDH-Whole seeds; DH-Dehulled seeds; 30- 30 Hz; 40- 40Hz; F- Fine fraction; C-Coarse fraction)

The above observations are consistent with those reported by Pulivarthi et al. (2025) for red lentils (24.3–16 µm), using the same instrument for analysis. It is evident that in fine fractions, particle size is reduced with the increasing wheel speed. This observation agrees with Funke, Loeffler, et al. (2022) and Pulivarthi et al. (2025), who studied lentils, and with many other studies investigating various legume protein ingredients. The separation of protein and starch granules during air classification is governed by a parameter termed as cut size. Cut size is the particle size at which there is a similar probability of a particle entering to either the fine or coarse fraction during a separation process and is known to be reduced at higher classifier wheel speeds (Schutyser et al., 2025). Accordingly, higher wheel speed stimulates higher protein concentration and the removal of starch in the fine fractions, as protein bodies are smaller than starch granules (Funke, Loeffler, et al., 2022).

The larger size of starch granules also explains the larger particle size of the coarse fractions, as evident through the scanning electron micrographs (**Figure 3.1**). However, the reported particle sizes of protein ingredients across studies are highly variable, largely influenced by the source material, processing conditions (Caldeira et al., 2024), and, especially, the drying method used for WE protein ingredients (Joshi et al., 2011).

3.3.3.2 Morphological Characteristics

Figure 3.1 represents the scanning electron microscopy images of lentil flour, fine and coarse fractions, and protein isolates. Ovoid and spherical-shaped starch granules are visible in the flour micrograph (**Figure 3.1b**), with protein bodies, fibers, and cellular materials adhered to them. These findings align with the observations of Caldeira et al. (2024) for lentil flour. It illustrates the diverse chemical composition of protein concentrates, which can significantly influence their techno-functional properties (Funke, Boom, et al., 2022).

Micrographs of fine fractions (**Figure 3.1c-f**) were characterized by a smaller number of starch granules and a more distinct presence of protein bodies. At the same time, small or broken starch fragments were observed in the micrographs and identified as residual starch in the fine fractions, in agreement with a previous study by Skylas et al, (2024). The lack of starch in the fine fraction micrographs further confirms that protein separation during the air-classification process is based

on particle size. Furthermore, it is apparent that the number of starch granules in the fine fractions decreases as the air classifier wheel speed increases.

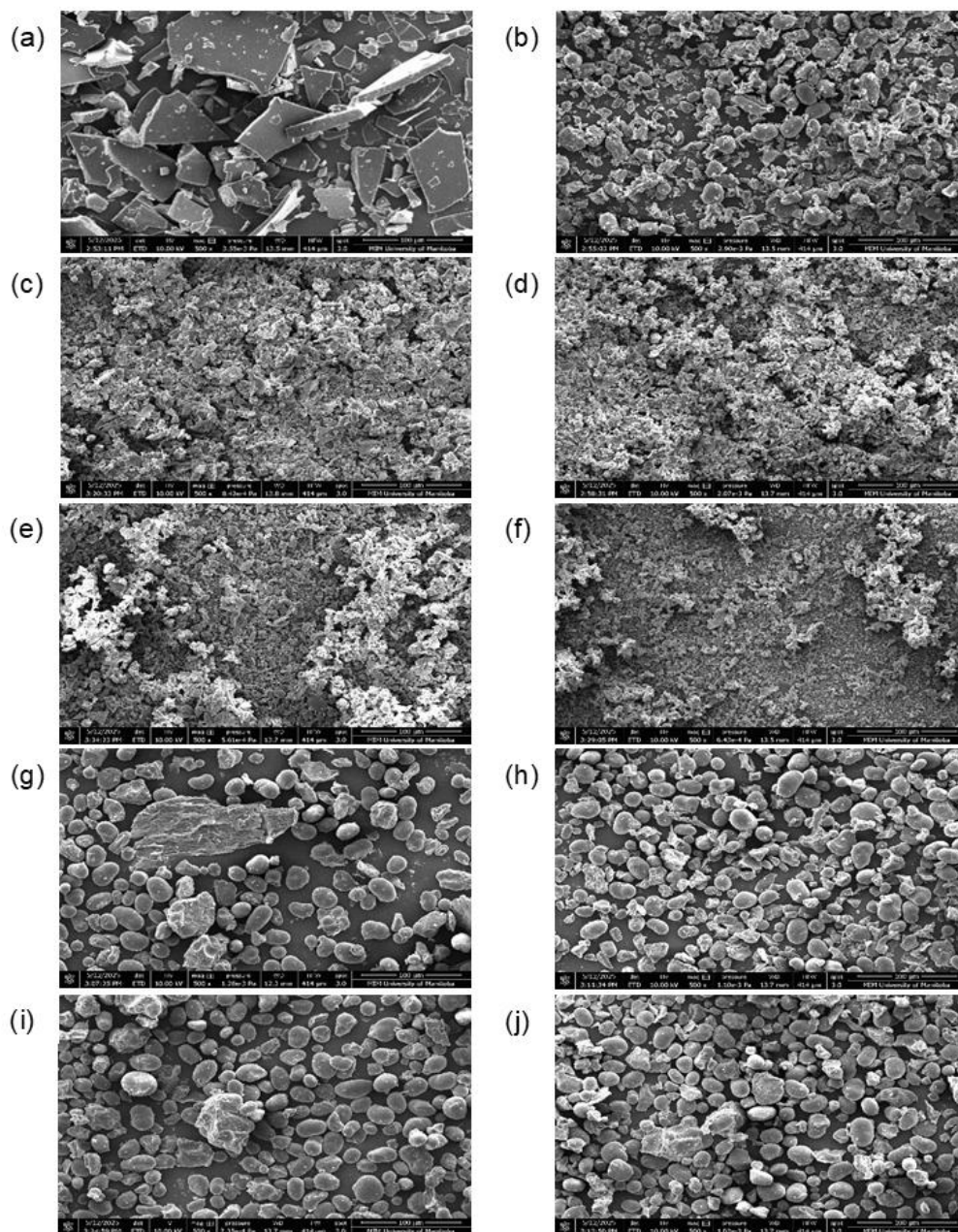


Figure 3.1: Morphological characteristics of (a) Protein isolate (b) Whole flour (c) NDH-30-F (d) NDH-40-F (e) DH-30-F (f) DH-40-F (g) NDH-30-C (h) NDH-40-C (i) DH-30-C (j) DH-40-C (amplification factor – x 500) (NDH-Whole seeds; DH-Dehulled seeds; 30- 30 Hz; 40- 40Hz; F- Fine fraction; C- Coarse fraction)

Micrographs of coarse fractions (**Figure 3.1g-j**) distinctively illustrated the abundance of starch granules which agreed well with the observations of Schlangen et al. (2022) related to mung bean, yellow pea, and cowpea flour. This characteristic is observed was all coarse fractions, regardless of the source material (Singh et al., 2024). Accordingly, scanning electron micrographs provided valuable information regarding the air classification separation mechanism, which primarily relies on particle size and density.

Micrographs of the protein isolate (**Figure 3.1a**) show flakes with a smooth surface. The smooth surface is induced by freeze-drying, whereas drying methods such as spray-drying can cause wrinkled surfaces (Caldeira et al., 2024). Joshi et al. (2011) also observed flake-like structure in protein isolates, but they appear to be thinner than those observed in the present study. Thus, the extraction method clearly affects the arrangement of proteins within the protein ingredients.

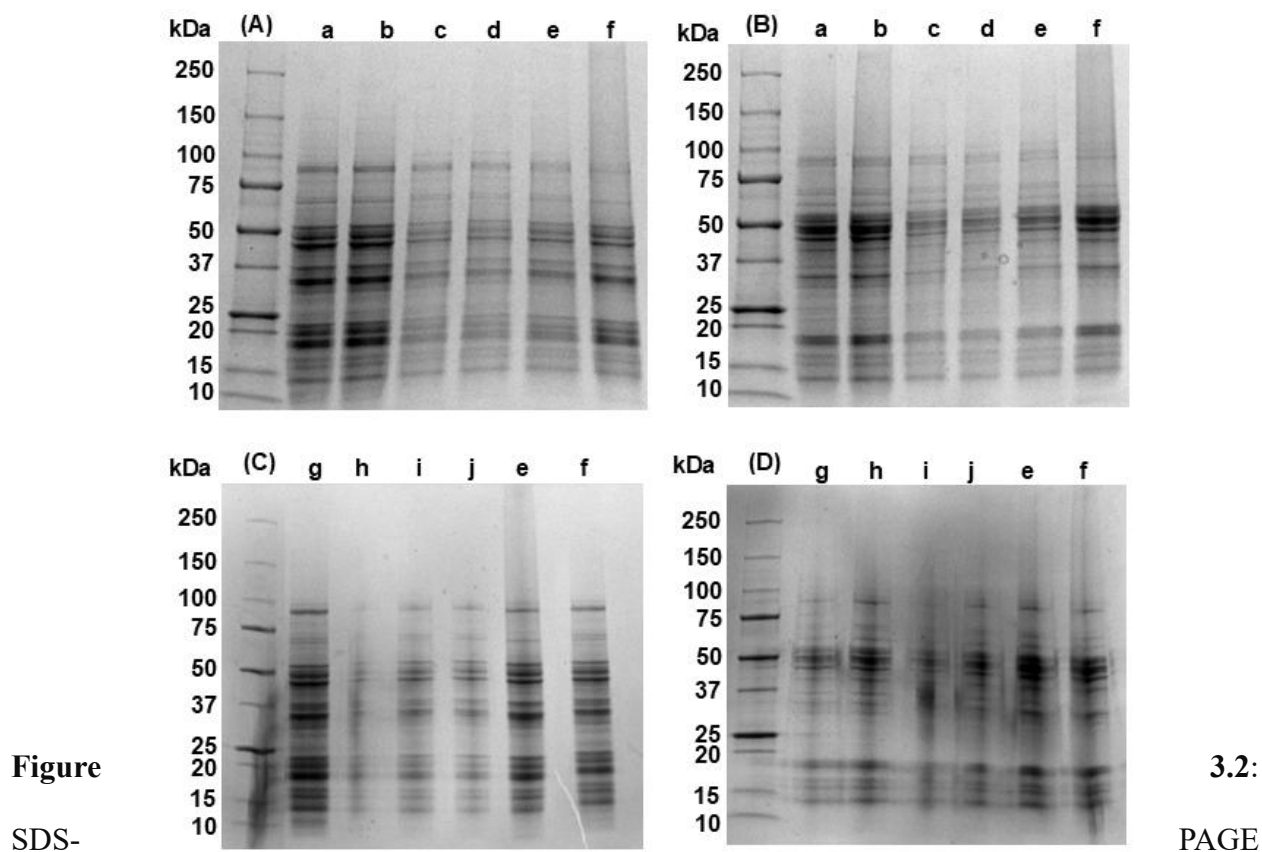
3.3.3.3 SDS PAGE Analysis

Non-reducing and reducing SDS-PAGE of the proteins in the fine and coarse fractions, protein isolate, and flour are shown in **Figure 3.2**. All these samples exhibited a complex protein profile, with many protein bands observed in the 12-85 kDa range. There was little difference observed between the protein profiles of the fine and coarse fractions and the isolate; however, band intensities differed. Accordingly, high-intensity bands were observed for fine fractions, particularly the protein isolate. The high intensity bands recorded for protein isolate could be due to its high purity and less interference on proteins by other components such as starch and fiber. It also became apparent that the DF protein samples showed a relatively higher number of protein bands than the protein isolate. This could be attributed to the milder processing conditions employed in dry fractionation compared with those used in wet extraction methods. Similar observations were made by Joshi et al. (2011), who evaluated the impact of different drying methods on the protein

composition of the protein isolates. The band patterns did not differ among the dehulled samples or the fractions produced at different wheel speeds, indicating that these factors do not significantly affect the protein profiles of the fine fractions. Therefore, the band patterns were analyzed in general.

Under non-reducing conditions, bands were noted at ~64, ~48, ~42, ~32, and 16-19 kDa. Bands appearing in the region 15-19 kDa can be related to the presence of gamma-vicilin and polypeptides formed as a result of storage protein cleavage (Ghumman et al., 2016). Bands observed at 60-64 kDa are attributed to the subunits of Legumin, which interact non-covalently to form the legumin protein (11S), a hexamer with a high molecular weight (380 kDa) (Miranda et al., 2022). Alonso-Miravalles et al. (2020) attributed the ~50 kDa bands to vicilin, which could also explain the intense bands around 48 kDa observed in the present study. Vicilin is a major globulin characterized as a 7S trimeric protein with a molecular weight of approximately 150 kDa. Barbana & Boye (2013) also identified vicilin-related bands in this region, attributing the band around 64 kDa to convicilin. Under reducing conditions, bands at 60-64 kDa partially disappeared, which is due to the disruption of disulfide bonds separating the acidic α -chain (40 kDa) and basic β -chains (20 kDa) (Miranda et al., 2022). Simultaneously, the intensities of the bands at 37 kDa and around 20 kDa increased, further confirming the formation of acidic and basic chains, respectively. Ladjal-Ettoumi et al. (2016) and Alonso-Miravalles et al. (2020) reported similar bands under reducing conditions, and both reduced the intensity of the band around 50 kDa, which was also observed in the present study. Formation of acidic polypeptides under reducing conditions was evident from bands at 2, 42, and 47 kDa (Jarpa-Parra et al., 2015). Accordingly, it is noted that

the inherent profile of lentil proteins is not significantly altered by any of the factors employed in the dry fractionation process.



profile of samples with reducing agent (A) and (C); without reducing agent (B) and (D); Lanes a: DH-30-F; b: DH-40-F; c: DH-30-C; d: DH-40-C; e: whole flour; f: Protein Isolate; g: NDH-30-F; h: NDH-40-F; i: NDH-30-C; j: NDH-40-C (NDH-Whole seeds; DH-Dehulled seeds; 30- 30 Hz; 40- 40Hz; F- Fine fraction; C- Coarse fraction)

3.3.3.4 Amino acid analysis

It is important to investigate whether dehulling or air classifier wheel speed has a distinct impact on amino acid composition, as this could influence functional properties, particularly those

mediated by the amino acids' hydrophobic or hydrophilic character. Considering the amino acid composition of each fraction obtained in this study (**Figure 3.3**), it is apparent that the major amino acids in lentils are glutamic acid, aspartic acid, arginine, lysine, and leucine. The limiting amino acids were identified as tryptophan and methionine, as reported by Kahraman (2016) for 6 different lentil varieties and Osemwota et al. (2022) for green lentil seeds.

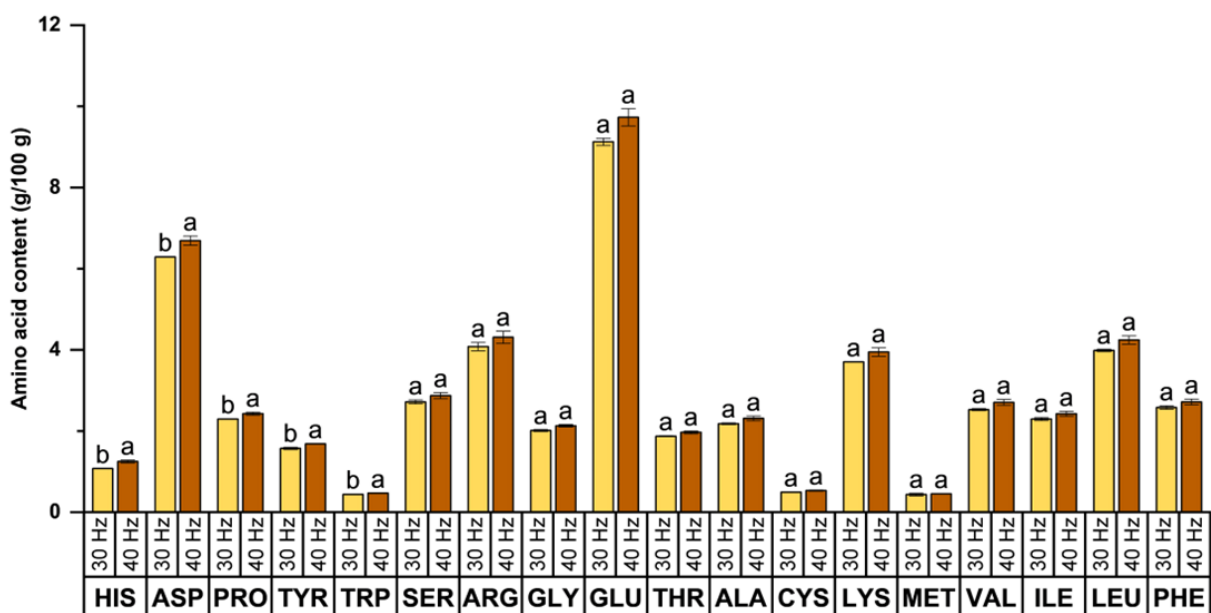


Figure 3.3: Effect of air classifier wheel speed on amino acid composition; lowercase superscripts – one-way ANOVA (n=2)

In the present study, dehulling was found not to significantly affect the amino acid composition of the fine and coarse fractions. However, it was determined that the air classifier wheel speed significantly affected several amino acids, namely histidine, aspartic acid, proline, tyrosine, and tryptophan (**Figure 3.3**). Each of these amino acids showed greater enrichment at higher wheel speeds. Among these, tyrosine and tryptophan have aromatic side chains and are higher in molecular weight. Schlangen et al. (2022) reported changes in the amino acid composition of the

fine fraction compared with flour following air classification, consistent with the current study's findings. Furthermore, this observation from the present study can provide insights into the protein fractions that participate in the fractionation process. Assessing the amino acid composition of the albumin, globulin, and glutelin fractions of green lentil seeds, Osemwota et al. (2022) recorded that tyrosine and tryptophan contents are significantly higher in albumins than globulins, and aspartic acid is the most abundant in all fractions. All these amino acids were among those that were significantly increased at higher wheel speeds. Therefore, it can be suggested that as wheel speed is increased, more albumin migrates towards the fine fraction, thus enhancing the protein enrichment. This is further supported by the smaller size and flexible shape of albumin compared to the larger, globular globulin (Shevkani et al., 2024). However, further studies are required to elucidate the factors governing amino acid enrichment during air classification.

3.3.3.5 Changes in protein secondary structures

Figure 3.4 shows the effect of air classification on the protein secondary structure components. The Amide I region is primarily associated with the C=O stretching vibrations of amide groups, along with in-phase N–H bending and C–N stretching motions (Yang et al., 2015). A prominent absorbance band was observed in the wavelength range 950–1050 cm⁻¹ for flour (Ahmad et al., 2020), which can be attributed to the presence of starch. At the same time, different bands that correspond to β sheet, α -helix, and β turns were identified upon the secondary derivative analysis. Based on available literature, extended chains are observed at 1600-1615 cm⁻¹; intermolecular β sheet at 1610–1627 cm⁻¹; β sheet structures at 1620-1642 cm⁻¹; random coils at 1640-1650 cm⁻¹; α -helix at 1650–1659 cm⁻¹; and β turns at 1660-1680 cm⁻¹ and 1694 cm⁻¹ (Yang et al., 2015; Nasrollahzadeh et al., 2023; De Angelis et al., 2024). β sheets are the most prevalent secondary

structure in legume proteins (Carbonaro et al., 2012), and this was also true for all the fine fractions, protein isolate, and flour samples analyzed in the present study.

It was also noteworthy that the relative content of α -helices decreased in the fine fractions obtained from the dehulled seeds. Simultaneously, an increase in β -sheet structures and aggregates was observed, indicating that mechanical forces applied during treatment altered the secondary structure and the extent of denaturation. Dehulled samples showed the presence of intermolecular β -sheets, in contrast to whole-seed samples, further supporting the conclusion that dehulling alters secondary structure, as proteins are subjected to greater mechanical forces when dehulled than when used as whole seeds. At the same time, the amino acid analysis depicted in **Figure 3.3** indicated a significant increase in the TYR and TRP contents, which are known to be present in the hydrophobic core of the protein molecules (Moinpour et al., 2020). It further confirms the changes in the protein structures, causing unfolding of protein molecules, exposing the hydrophobic sites of proteins. Furthermore, the reduction in α -helices can also be correlated with the effect of mechanical forces in inducing denaturation as Nasrollahzadeh et al. (2023) also recorded a decline in the α -helices upon extrusion in the legume proteins, which was attributed to an increase in disordered structures.

Information on the secondary-structure analysis of lentil protein concentrates is very limited in the literature, making it difficult to compare and validate results. Hence, further studies are required to assess the effects of processing methods and varietal differences on the secondary structures of lentil proteins.

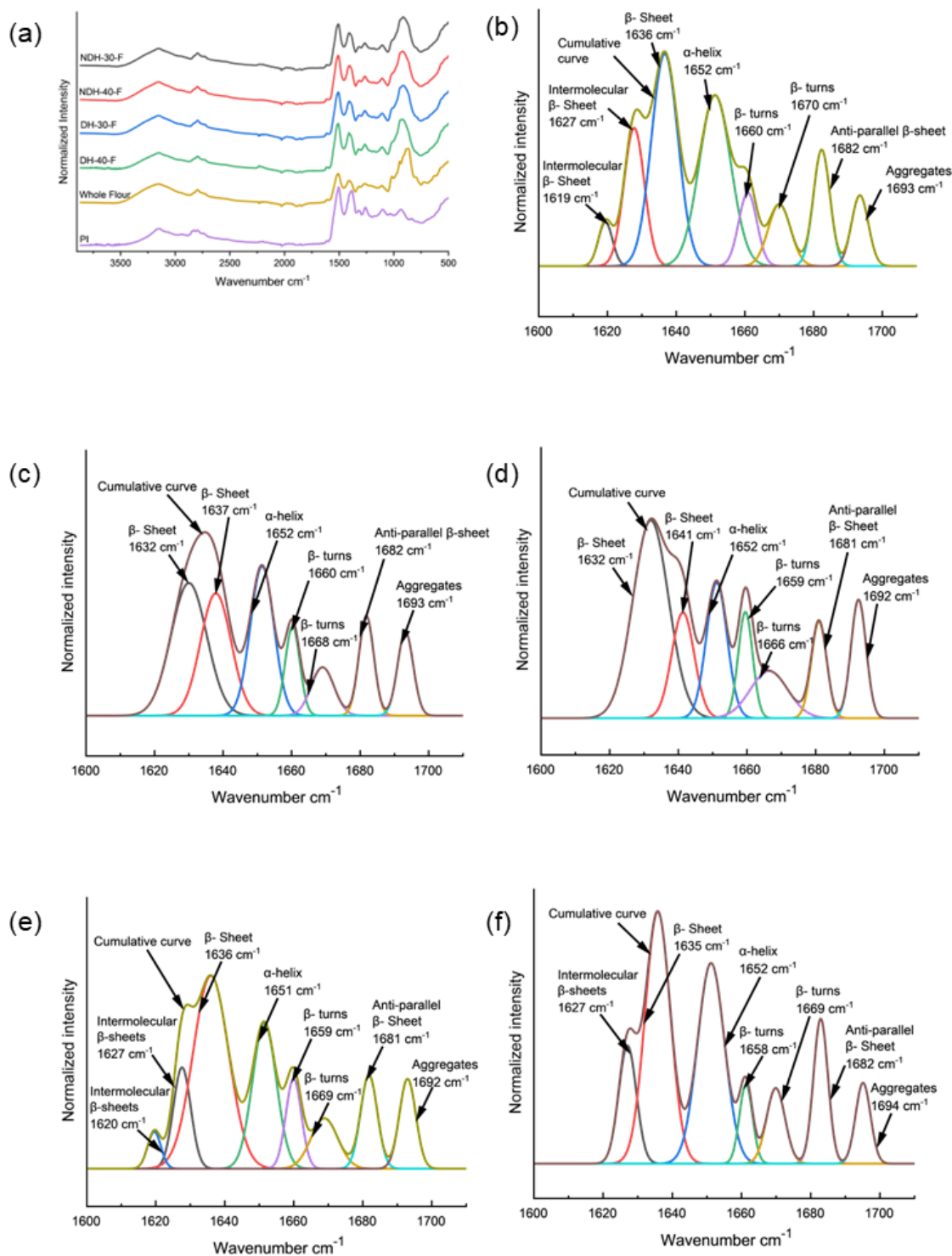


Figure 3.4: Protein structural changes in fine fractions (a) FTIR spectra for lentil flour, fine fractions, and protein isolate, (b–d) Peak fitting of Amide I with the relative proportion of each secondary structure of (b) Lentil flour (c) NDH-30-F, (d) NDH-40-F (e) DH-30-F (f) DH-40-F

3.3.3.6 Total Phenolic and Phytic Acid Content

The Total Phenolic Content (TPC) and phytic acid content of the concentrates are indicated in **Figure 3.5a and 3.5b**, respectively. The TPC contents of the protein ingredients were within the range 0.86-1.20 mg GAE/ g dw, whereas dehulling caused a significant reduction. At the same time increased wheel speed caused a relative enrichment of total phenolic content. Polyphenols are often identified as bioactive compounds yet are also categorized as antinutritive compounds owing to their affinity for and interactions with proteins via sulfhydryl groups and free amino acids. This negatively affects the digestibility and bioavailability of proteins in food systems (Sęczyk et al., 2019).

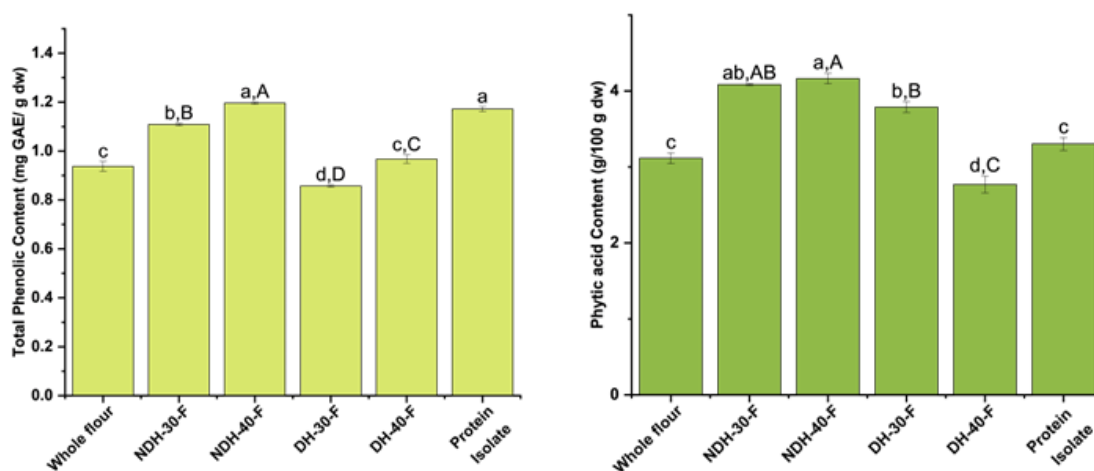


Figure 3.5: Phytochemical composition (a) Total Phenolic Content (b) Phytic Acid Content of fine fractions compared with controls (Uppercase superscripts – two-way ANOVA; lowercase superscripts – one-way ANOVA) (NDH-Whole seeds; DH-Dehulled seeds; 30- 30 Hz; 40- 40Hz; F- Fine fraction)

Han et al. (2025) assessed the TPC content of different red lentil fractions and recorded values within a similar range as above, and similar variations between the wet and dry extracted protein fractions. The effect of dry fractionation in enriching fine fractions with polyphenols has been

confirmed by several studies across various legumes, such as faba bean and red lentils (Skylas et al., 2023), and rapeseed and sunflower meals (Laguna et al., 2018). Among all, it was noted that enrichment levels were highly dependent on the raw material (Han et al., 2025) and on the dry fractionation mechanism (Laguna et al., 2018). It is also evident that most free phenolics are associated with the protein particles, thus they move to the fine fraction upon fractionation. Phenolic compounds tend to make complexes with proteins by interacting reversibly through hydrogen bonds, or irreversibly by oxidation into quinones, which can favor their separation into the fine fraction during the air classification process, along with the proteins (Hernández-Jabalera et al., 2015). Furthermore, the significant reduction in TPC with dehulling also indicates that it can be used as an effective mechanism to reduce enrichment by removing the polyphenols present in the seed coat (Fernando, 2021).

The phytic acid content of the samples ranged within 2.77 – 4.17 g/ 100 g dw. Air classification caused a significant enrichment in phytic acid content, yet the effect was significantly lower when the seeds were subjected to dehulling. Phytic acid is categorized as an antinutrient due to its action towards hindering the absorption and bioavailability of mineral ions, thus causing mineral deficiencies (Samtiya et al., 2020).

The enhancing effect on the phytic acid content during dry fractionation was similarly observed by Han et al. (2025) for red lentils, and (Saldanha do Carmo et al., 2020) for yellow peas and faba beans. The authors suggested that phytic acid behaves similarly to phenolic compounds, as it tends to associate with proteins and move to the fine fraction during fractionation. The enrichment of phytic acid during air classification is considered a disadvantage, as it can induce unfavorable changes in the taste and nutritional value of the protein ingredients, along with other antinutritional factors (Schutyser et al., 2015). Therefore, it is important to employ different pretreatment

methods, namely, physical treatments such as soaking and dry heat, or enzymatic treatments such as lactic acid fermentation (Schutyser et al., 2015). However, the number of studies directly assessing the effect of air classification on phytic acid is limited. Thus, further assessment is needed to establish the correct mechanistic explanations in this context.

3.3.4 Effect of Air Classification Conditions and Dehulling on Techno-functional Properties

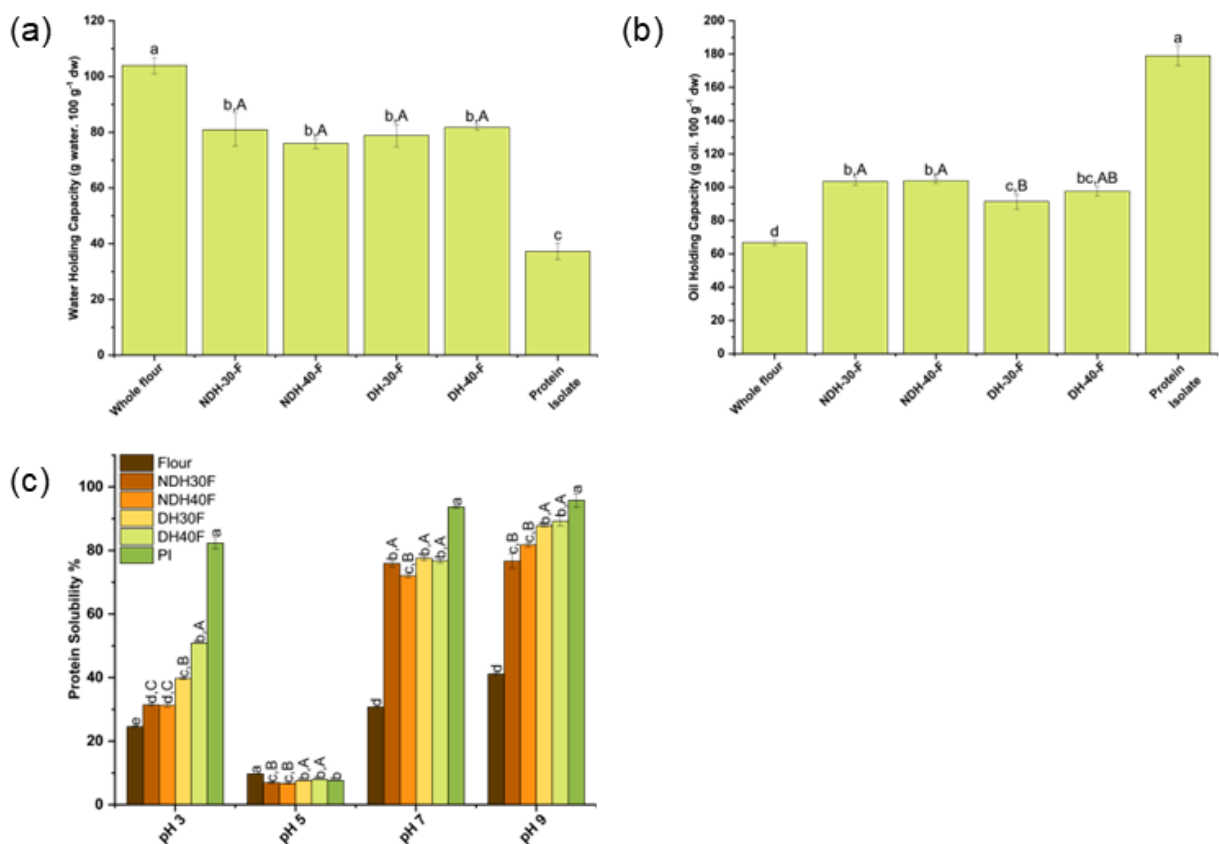


Figure 3.6: Techno-functional Properties of fine fractions compared with controls (a) Water holding capacity (b) Oil holding capacity (c) Protein solubility (Uppercase superscripts – two-way ANOVA (n=3); lowercase superscripts – one-way ANOVA (n=3)) (NDH-Whole seeds; DH-Dehulled seeds; 30- 30 Hz; 40- 40Hz; F- Fine fraction)

Assessing the techno-functional properties of legume proteins is critical for understanding how their molecular composition and structural organization can be effectively utilized during food

formulation and processing. Therefore, several important functional properties were assessed, and efforts were made to relate them to the structural and functional characteristics of the protein concentrates.

3.3.4.1 Water Holding Capacity (WHC)

Figure 3.6a indicates the effect of air classifier wheel speed and dehulling on WHC. Recorded values were within the range 37.2 – 103.9 g oil. 100 g⁻¹ dw, with lowest value recorded for protein isolate and highest for whole flour. Air classification reduced WHC compared with whole flour, regardless of the effects of either variable. This follows a trend similar to that of many other air classified pulse proteins as observed by Saldanha do Carmo et al. (2020), Oluwajuyitan & Aluko (2025) and Caldeira et al. (2024). Simultaneously, higher WHC was recorded in coarse fractions (Saldanha do Carmo et al., 2020) which was attributed to the enrichment of fiber and starch in coarse fractions. Accordingly, lower WHC of fine fractions compared to whole flour can be assumed to be influenced by the chemical compositional changes caused by air classification. Saldanha do Carmo et al. (2020) In addition, the reduced WHC of protein concentrates could also be correlated with levels of denaturation caused by mechanical forces applied during dehulling and air classification. Denaturation is associated with enhanced exposure of hydrophobic groups, resulting in reduced WHC (Rahim et al., 2023). Secondary structure analysis (**Figure 3.4**) provides evidence to the levels of denaturation through increased levels of β -turns and aggregates, which could partly explain the above observations.

The WHC recorded for protein isolate in the present study was significantly lower than that of the protein concentrates. During alkaline extraction, materials are subjected to extreme pH conditions, which causes high levels of denaturation, resulting in increased hydrophobicity of proteins due to exposed hydrophobic groups (Rahim et al., 2023) . This could have affected the lower WHC of

protein isolate, which was also true for a comparison between commercial protein concentrates and isolates conducted by De Angelis et al. (2024). Values recorded by Joshi et al. (2011) for WHC of alkaline-extracted PI was in between 43 – 48 g water. 100 g⁻¹ dw, which is highly agreeable with the value recorded in the present study. De Angelis et al. (2024). However, reviewing the WHC values recorded in past studies for alkaline-extracted PI, Caldeira et al. (2024) recorded a large range from 43 to 350 g water. 100 g⁻¹ dw, indicating the effect of variety, processing methods, etc., on WHC of lentil protein ingredients. (Caldeira et al., 2024)

3.3.4.2 Oil Holding Capacity (OHC)

The effect of air classification parameters on OHC is indicated in **Figure 3.6b**. OHC values of the samples were found to be within the range 66.6 – 178.9 g oil. 100 g⁻¹ dw, with the lowest value for whole flour and the highest for protein isolate. An opposite pattern to WHC was identified for OHC, where protein ingredients recorded higher values than the flour and coarse fractions. OHC values recorded in this study closely align with those reported by De Angelis et al. (2024) of 1.02 g oil. 100 g⁻¹ dw and 1.77 g oil. 100 g⁻¹ dw for commercial concentrates and isolates, respectively. Increased OHC in protein ingredients is consistent with the findings of Saldanha do Carmo et al. (2020) and is explained by the hydrophobic nature of legume globulins, which are the key proteins in legumes. In general, the enhanced OHC could also be associated with the increased exposure of hydrophobic groups resulting from protein denaturation as discussed in section 3.4.4.1. These findings can be further supported by the relative increase in hydrophobic amino acids as recorded in **Figure 3.3**. Moreover, OHC of alkaline-extracted PI was significantly higher than that of the concentrates, suggesting that the proteins in the isolate are more hydrophobic. The relatively higher OHC in PI could be attributed to the higher protein content in PI.

Another noteworthy observation in the present study is that the fine fractions obtained from whole seeds (NDH) exhibited significantly higher OHC than those from dehulled seeds. This could be stimulated by the enhanced fiber content in the concentrates from whole seeds, facilitating better oil retention (Wang et al., 2020). Accordingly, protein concentrates exhibit appreciable levels of WHC and OHC, thereby expanding their applications to various food products, including breads, doughs, pastes, meat analogues, and other textured foods.

3.3.4.3 Protein Solubility

The interplay between protein-protein and protein-solvent interactions influences protein solubility. **Figure 3.6c** shows the effect of air classification process parameters on protein solubility at different pH values. Across all samples, solubility was lowest at pH 5 and increased under both acidic and alkaline conditions. Lower solubility at pH 5 can be explained by the fact that it is close to the isoelectric pH (pI) of lentil proteins, which minimizes electrostatic repulsion and thereby reduces solubility due to protein-protein interactions (Miranda et al., 2022). Globulin, the most abundant protein fraction in lentils, has a pI around pH 5, leading to its precipitation at that pH (Yang et al., 2023). At this pH, soluble protein content mainly consists of albumin, which is less abundant, suggesting lower protein solubility. The increase in solubility at acidic and alkaline conditions can be attributed to the surface charges of the proteins, which result in electrostatic repulsions between the proteins and protein-water interactions, thereby favoring their solubility (Pulivarthi et al., 2025). At acidic pH values, carboxyl and amine groups on amino acid side chains are protonated, resulting in positive surface charges (Shen et al., 2020), whereas at alkaline pH values, amino acid side-chain ionization results in negative surface charges (Oluwajuyitan & Aluko, 2025).

A significant effect of dehulling towards enhancing solubility was observed at pH 3 and 9. Increased solubility in dehulled samples was reported in a similar study that employed fava bean protein isolates (Kamani et al., 2024) and can be explained by possible complex formation between proteins and phenolic compounds (Sęczyk et al., 2019). As indicated in **Figure 3.5**, dehulling significantly reduces the total phenolic content, which could have reduced the formation of such complexes, altering the surface charges of protein molecules, and negatively impacting solubility. Moreover, the significant effect of increased wheel speed on protein solubility at acidic pH can be attributed to several factors. At increased wheel speeds, protein enrichment is enhanced (**Table 3.1**) and particle size is reduced (**Table 3.2**) which are factors that essentially contributed to increased solubility.

3.3.4.4 Emulsifying Activity Index (EAI) and Emulsion Stability (ES)

Figure 3.7a and **3.7b** represent the effects of air classifier wheel speed and dehulling towards EAI and ES, respectively. Emulsifying properties are another important functional property discussed in relation to the protein ingredients. EAI is an indication of the amount of oil emulsified by a given amount of protein (Lam & Nickerson, 2013). EAI and ES values were enhanced by dehulling and higher wheel speed at acidic pH, whereas no significant effects were identified at other pH values. Emulsifying properties of fine fractions were better than the coarse fractions, and was observed to be higher at higher pH values. Many factors related to denaturation, surface charge, protein solubility, etc. can impact such changes. The above trend for EAI and ES is consistent with the findings of Caldeira et al. (2024).

Generally, PI showed relatively high ESI, which may be attributed to denaturation, which enhances protein binding at the interface. Alkaline conditions during extraction expose hydrophobic groups in the protein structure, thereby increasing hydrophobicity and contributing to enhanced EAI and

ESI (Karaca et al., 2011). Better emulsifying properties of fine fractions when compared to coarse fractions, suggest that starch has weaker emulsifying properties than proteins (Oluwajuyitan & Aluko, 2025). On the other hand, EAI for emulsions formed at alkaline pH values, especially at pH 9, was higher than that at pH 3 and 5, which provided evidence for the positive influence of protein solubility on the EAI (Oluwajuyitan & Aluko, 2025).

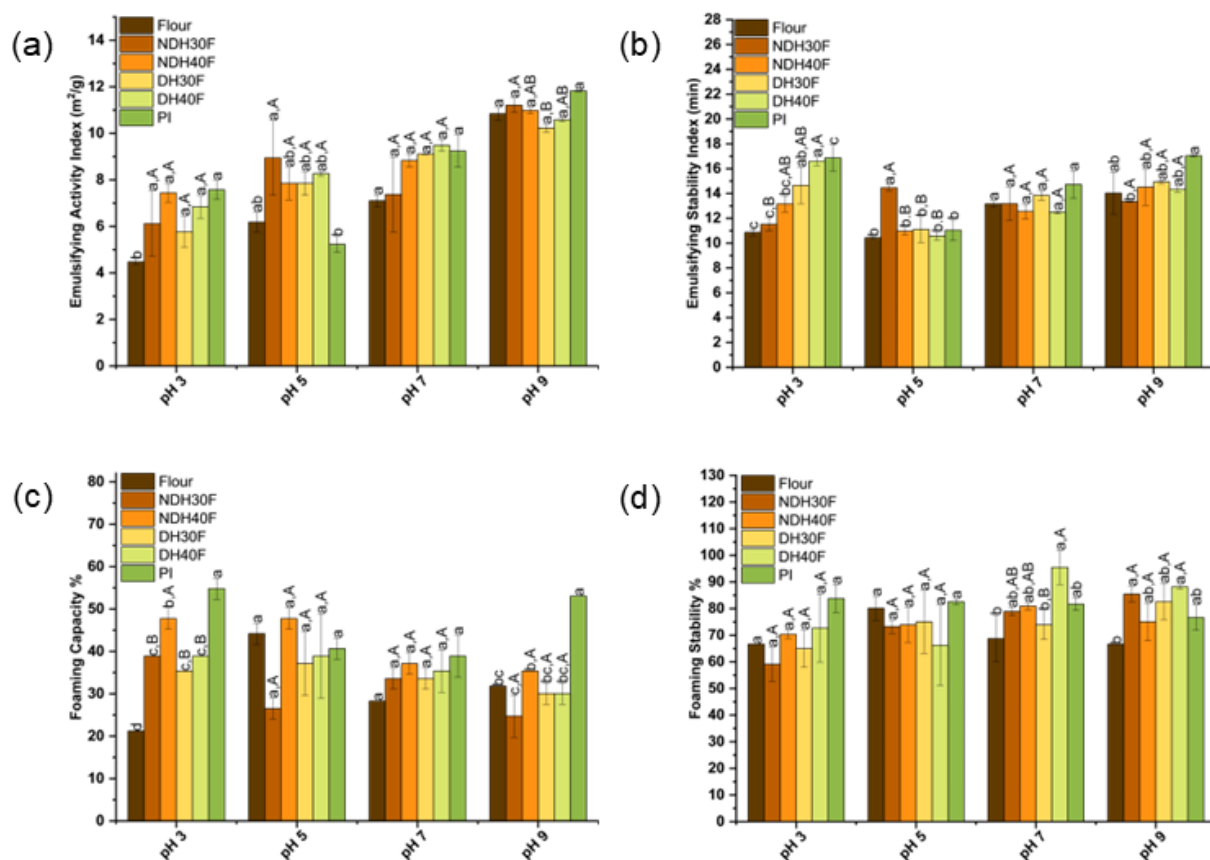


Figure 3.7: Techno-functional Properties of fine fractions compared with controls (a) Emulsifying activity index (b) Emulsifying stability index (c) Foaming capacity (d) Foaming stability (Uppercase superscripts – two-way ANOVA (n=3); lowercase superscripts – one-way ANOVA (n=3)) (NDH-Whole seeds; DH-Dehulled seeds; 30- 30 Hz; 40- 40Hz; F- Fine fraction)

3.3.4.5 Foaming Capacity (FC) and Foaming Stability (FS)

Figure 3.7c and **3.7d** represents the FC and FS values of the protein ingredients at different pH values and no significant effects of dehulling or wheel speed were identified except in FC at pH 3. Air classification significantly improved FC at pH 3. This is consistent with the findings of Saldanha do Carmo et al. (2020), who studied the properties of faba bean concentrates. The values recorded in the present study are consistent with those reported by Kaur & Sandhu (2010) for green lentils, which ranged from 33.9–47.3%.

It is also interesting to note that, despite the relatively lower protein content, fine fractions have FC and FS values that are similar to or higher than those of PI. Many authors have suggested explanations for the higher FC and FS in fine fractions compared with WE protein isolates. J. Yang et al. (2023) propose that albumin-type proteins are better preserved in the fine fractions during dry fractionation, which contributes to higher FC and FS. Fine fractions contain carbohydrates in addition to proteins, which may be beneficial for foaming, thereby enhancing FC and FS (Silventoinen et al., 2018). A comparable pattern was observed in red lentils as well, attributed to the ability of dry fractionation to better preserve the native functionality of the proteins than wet fractionation (Pulivarthi et al., 2025). FC and FS are important functional properties that support the use of protein concentrates in aerated foods, such as ice cream, soft doughs, and confectionery.

3.3.5 In Vitro Protein Digestibility (IVPD) and PDCAAS Values

Assessing in vitro protein digestibility is widely employed to estimate the extent to which proteins can be enzymatically hydrolyzed under simulated gastrointestinal conditions, providing insight into their potential nutritional quality and bioavailability (Santos-Sánchez et al., 2024). **Figure 3.8** illustrates the effect of air classification process parameters towards the IVPD values of lentil protein ingredients.

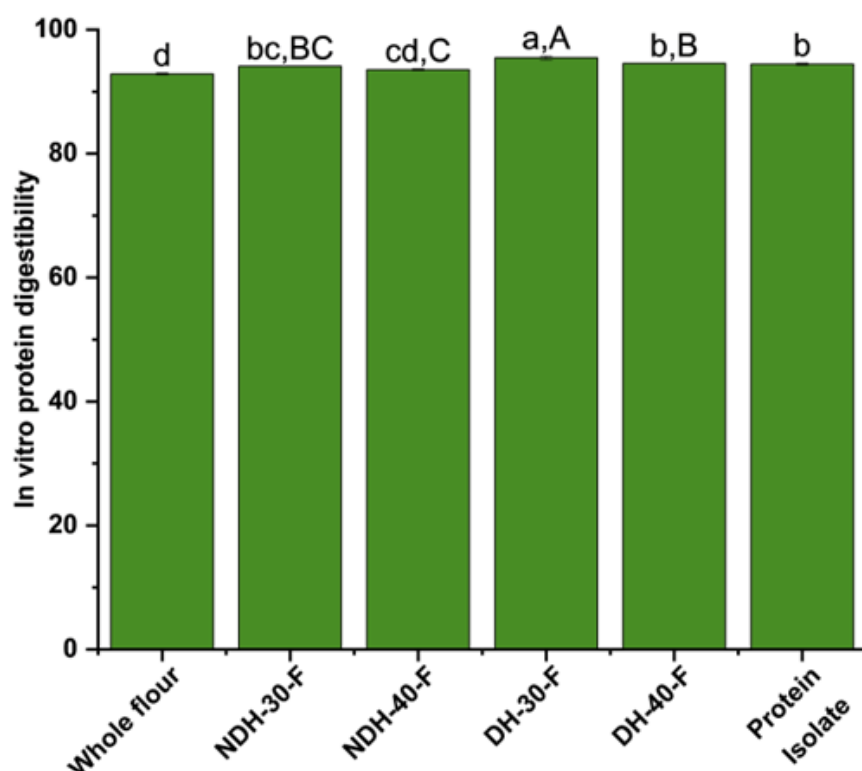


Figure 3.8: In Vitro Protein Digestibility (IVPD) values of fine fractions compared with controls (Uppercase superscripts – two-way ANOVA; lowercase superscripts – one-way ANOVA) (NDH- Whole seeds; DH-Dehulled seeds; 30- 30 Hz; 40- 40Hz; F- Fine fraction)

It was unveiled that dry fractionation significantly enhances protein digestibility, and this effect can be further enhanced by dehulling as a pretreatment. It aligns with the findings of related studies, such as Alonso et al. (2000) for faba and kidney beans, and Bishnoi & Khetarpaul (1993) for different pea varieties. Dehulling reduces the fibre-containing cell wall material, which may explain the above observation. Several studies report that higher fiber content can reduce the accessibility of proteolytic enzymes, thereby reducing digestibility (Santos-Sánchez et al., 2024). An increase in IVPD was also observed with reduced phytic acid in the concentrates, suggesting that phytic acid negatively interferes with digestibility. Similar findings were ported by Chitra et al. (1996) for chickpea, pigeon pea, mungbean, and soybeans, and by Rosa-Sibakov et al. (2018)

for faba beans. Many antinutritional factors, including phytic acid, form complexes with proteins through various interactions, which increase cross-linking and make the proteins less accessible to proteolytic enzymes (Alonso et al., 2000). It is evident that the number of studies investigating the effects of air-classification processing parameters is limited; thus, further exploration is needed to identify ways to improve the digestive properties of protein concentrates through dry fractionation.

Table 3.3: Digestibility and PDCAAS values of protein concentrates and controls

Sample	LEAA ^a	IVPD ^b	PDCAAS ^c
Whole Flour	TRP	0.93 ^d	0.66
NDH-30-F	MET+CYS	0.941 ^{bc}	0.70
NDH-40-F	MET+CYS	0.935 ^{cd}	0.71
DH-30-F	MET+CYS	0.954 ^a	0.67
DH-40-F	MET+CYS	0.945 ^b	0.67
Protein Isolate	MET+CYS	0.944 ^b	0.69

(NDH-Whole seeds; DH-Dehulled seeds; 30- 30 Hz; 40- 40Hz; F- Fine fraction ^aLimiting essential amino acid; ^bIVPD = In vitro protein digestibility; ^cPDCAAS = AAS*IVPD

Based on the IVPD values and the amino acid composition, the PDCAAS values were calculated. **Table 3.3** presents the calculated values to be within the range 0.66 to 0.71, which indicates moderate protein quality of the protein ingredients. According to literature, most Canadian pulses have recorded PDCAAS values above 0.5, whereas green lentils have recorded relatively higher PDCAAS value of around 0.63 (Nosworthy et al., 2017). Accordingly, the values recorded in the present study is in high agreement with the available research data. The detailed calculation of

PDCAAS values is indicated in **Supplementary Material 1: Amino acid composition and PDCAAS calculation of fine fractions compared with controls**

The limiting amino acid for all the processed samples was Sulphur containing amino acids. Amino acid composition of the samples (**Figure 3.3**) confirmed the significant improvement of tryptophan during air classification, as a result of protein unfolding encouraged by partial denaturation. It has also impacted the PDCAAS values, by improving the values and thus the protein quality. Despite recording significantly higher IVPD values, dehulled samples recorded lower PDCAAS values due to lower MET and CYS contents, which were identified as the limiting amino acids. The reduced sulphur containing amino acid content in dehulled samples can be attributed to the protein aggregate formation as confirmed by the secondary structure analysis as indicated in **Figure 3.4**. During dehulling, samples were subjected to further mechanical stress than other samples, causing an increase a higher level of aggregate formation. Formation of disulphide bonds and sulphur cross links through methionine and cysteine oxidation is a predominant phenomenon associated with protein aggregate formation (Tang et al., 2009). This reduces the free sulphur containing amino acid content in the sample, thereby influencing the digestibility and PDCAAS values.

The above observations highlight the need for complementing the lentil protein ingredients with a protein source rich in sulphur containing amino acids, to ensure adequate nutritional and digestibility properties.

3.4 Conclusion

This study demonstrates that dehulling and air classifier wheel speed significantly influence the compositional, structural, and functional characteristics of green lentil protein concentrates. While air classification parameters such as protein yield and separation efficiency remained unaffected, both dehulling and increased wheel speed increased protein and fat content, particle-size

distribution, amino acid profile, and digestibility. The techno-functional properties, including solubility, emulsification, foaming, and gelation, showed pH-dependent responses to the processing variables. These findings highlight the importance of optimizing dehulling and wheel speed settings to tailor lentil protein ingredients for specific food applications. These findings suggest that the desired structural and functional properties of the concentrates can be achieved by appropriately adjusting processing parameters, including pretreatments such as dehulling and air classifier wheel speed. Overall, air classification is a sustainable and effective method for producing high-quality plant protein concentrates, with tunable properties achievable through controlled processing conditions.

Author contributions

Shimani Attygalle: Conceptualization, data curation, formal analysis, methodology, software, validation, visualization, writing-original draft, writing-review and editing

Nandika Bandara: Conceptualization, funding acquisition, investigation, methodology, project administration, resources, software, supervision, editing

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Chapter 4: Enhancing the Functionality of Lentil Protein Concentrates Using High Voltage Atmospheric Cold Plasma (HVACP)

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Abstract

Many plant protein ingredients exhibit limited techno-functional properties, necessitating enhancement through various modification technologies. High-voltage atmospheric cold plasma (HVACP) is an emerging non-thermal modification technology, that is being explored for improving protein functionality. This study optimized high-voltage atmospheric cold plasma (HVACP) modification of lentil proteins using a dielectric barrier discharge (DBD) plasma system. The treatment conditions were tailored for lentil protein concentrates, and their effects on compositional, structural, thermal, and techno-functional properties were systematically evaluated. Treatment time and voltage combinations were optimized to increase protein solubility and reduce carbonyl formation, followed by analysis of the amino acid profile, secondary structure, thermal behavior, functional properties, antinutritional factors, and in vitro protein digestibility. Four optimal conditions (1, 2, 5 min/15 kV, and 1 min/20 kV) were identified. HVACP treatment

significantly enhanced functional properties, including improved protein solubility at pH 3 and approximately two-fold higher oil-holding capacity, while water-holding capacity remained unchanged. Emulsifying and foaming properties were largely unaffected, except for reduced stability at pH 3. Structural analysis revealed increased β -sheet content and protein aggregate formation in treated samples, indicating partial denaturation. Treated proteins showed no changes in molecular weight distribution. Treated samples exhibited altered morphology, increased total phenolic content, improved in vitro protein digestibility, and no significant change in phytic acid content. Additionally, when HVACP was applied for *E. coli* reduction, it demonstrated dual functionality, achieving a 2 log CFU/g reduction per cycle at 20 kV. Overall, HVACP demonstrated very high potential in improving protein concentrates, ensuring their wide applicability in various food applications while overcoming its functionality challenges.

Keywords – DBD Cold Plasma, Protein concentrates, optimization, dual functionality

4.1 Introduction

Increasing global population and various technological and socioeconomic factors have caused significant changes in dietary patterns worldwide. Global protein consumption is continuously on the rise, creating increased demand for animal as well as plant protein sources (Khanashyam et al., 2025). Plant protein ingredients are gaining popularity due to the emergence of different dietary patterns such as vegan, vegetarian, and flexitarian, and also due to the increased use of them in natural products (Ismail et al., 2020). Plant protein ingredients are mainly obtained from various cereals and pulses, and numerous wet and dry extraction techniques are employed in their production. These ingredients demonstrate a great diversity in terms of their chemical and structural properties, which also determines the techno-functional properties associated with their applications in food products (Messina et al., 2025). Despite numerous advantages such as accessibility and sustainability, plant protein ingredients often meet with a challenge associated with relatively poor functionality, which often restricts their utilization in commercial applications (Kong et al., 2025). Therefore, many present studies focus on developing and optimizing protein modification technologies, which can help to overcome this barrier and expand the applicability of plant protein ingredients within the food industry.

Modification technologies are broadly classified as chemical, physical, and biological based on their approaches. Biological methods are mostly enzymatic, and proceed with precision and specificity, yet are associated with limited applications (Zink et al., 2016). Chemical modification approaches are advancing day by day and are widely used in industrial settings due to the affordability and convenience (Zhao et al., 2025). Physical modification technologies have been in use for a long period of time, and recent advances are directed towards exploring technologies that cause minimum harm to native protein structure and functionality. This has paved the way for

the emergence of numerous nonthermal protein modification methods (Mirmoghadaie et al., 2016).

High Voltage Atmospheric Cold Plasma (HVACP) is a novel, nonthermal processing method that is increasingly used in the food industry. It operates on plasma, the fourth state of matter, produced by ionizing a carrier gas to form a mixture of reactive species, such as photons, electrons, free radicals, and ions, by providing sufficient energy (Olatunde et al., 2023). Cold plasma can be generated at relatively low pressures and power levels, making it well-suited for food applications because it causes minimal thermal damage to food components (Rout & Srivastav, 2024). (Bahrami et al., 2016). However, for several reasons, HVACP technology is not universally applicable across different protein sources and requires optimization of operating conditions based on the specific composition and the desired functional outcome. One main reason for the need for optimization is the diverse structural and compositional factors of various grain sources. Grains are broadly classified as oilseed grains (eg: canola, flaxseed, soy, lupin, etc.) that are rich in lipids, and legumes (eg: lentil, pea, faba bean, etc.) that are rich in carbohydrates. For example, these compositional differences elicit distinct responses when subjected to atmospheric cold plasma treatment (HVACP) due to their interaction with the reactive species generated. Rout & Srivastav (2024) assessed the impact of HVACP on soy and pea protein isolates and observed that the two isolates responded differently in terms of Trypsin Inhibitory activity and in vitro digestibility, indicating that optimized conditions vary across the grains. Similar observations were also reported by Wu et al. (2023) for mung bean, kidney bean, and adzuki bean, with significant differences in secondary-structure alterations among the three legume species under the same HVACP treatment. Processing parameters are another factor that largely determines HVACP treatment efficiency. It is mostly correlated with the energy of HVACP incident on the samples, as represented by the

atmospheric plasma dose. This parameter is highly influenced by factors such as voltage, power, frequency, and treatment duration. At the same time, the feed gas also plays a significant role in determining the impact of HVACP treatment, as noble gases preserve the biochemical and physicochemical attributes yet require longer treatment durations, whereas in contrast, reactive gases such as nitrogen and oxygen cause a more sterilization effect at a shorter treatment duration with a considerable impact on the biochemical composition of the food material (Olatunde et al., 2023).

An emerging trend associated with HVACP is its use to enhance the techno-functional properties of protein ingredients. The wet extraction method is the most widely used for producing protein ingredients. However, it is associated with several disadvantages, including the generation of wastewater and chemical solvents, and the loss of natural structure and function due to denaturation caused by high temperatures and pH conditions (Schutyser et al., 2025). Dry fractionation is a more sustainable alternative that completely avoids the use of water and chemical solvents, and is mainly carried out through air classification. Air classification is a process that separates the protein and starch molecules based on their size and density through an interplay between the drag force caused by the air stream and the centrifugal force (Schutyser et al., 2025).

Reviewing a large number of related studies, Olatunde et al. (2023) indicated the potential of HVACP as a promising technology to enhance functionality of wet extracted protein ingredients, yet a significant research gap remains regarding dry fractionated protein concentrates. Therefore, bridging this gap, the present study tested the hypothesis HVACP treatment can be effectively utilized to enhance the compositional, techno functional, and digestibility of dry fractionated protein concentrates. The study was conducted with the aims of optimizing the HVACP process to

improve techno-functional properties and digestibility of protein concentrates, and exploring the dual function of HVACP in ensuring the microbial safety of protein ingredients.

4.2 Materials and Methods

4.2.1 Sample Preparation

4.2.1.1 Air classification

Laird green lentils (*Lens culinaris*) were purchased from AGT Foods (Regina, SK, Canada). Lentils were dehulled using a Rice Whitener (RMB 10G, SATAKE Group, USA), milled using a rotor beater mill (SR 300, Retsch GmbH, Germany), and subjected to air classification using a Prater REMA Classifier (Prater Sterling, Illinois, USA) at an air classifier wheel speed of 40 Hz (5723 rpm). The fine fractions obtained were collected and stored at 4 °C for further analysis.

4.2.1.2 Alkaline Extraction of Protein Isolate

Protein isolate (PI) obtained by conventional alkaline extraction served as the control in the study. Alkaline extraction was performed according to the method described by Karaca et al. (2011), with some modifications. Lentil flour (<0.2 mm) was dispersed in Milli-Q water in a 1:10 (w/v) ratio and thoroughly mixed for 10-15 sec. Following homogenization (Model 850, Fisher Scientific, Ottawa, ON, Canada) at 14,000 rpm for 1 min, the solution was adjusted to pH 9 with 5 M NaOH, and protein was extracted (250 rpm, 2 hrs, room temperature). The obtained alkali extract was centrifuged (12,1000 g, 30 min, 4 °C), and the supernatant pH was adjusted to 4.5 with 2.5 M HCl. Precipitation was allowed to occur overnight at 4°C, and the protein precipitates were collected. Pellets were then washed twice with the same centrifugation step as above. Pellets were then redispersed in water, the pH adjusted to 7, and the mixture freeze-dried at -21 °C (HRFDXL, Harvest Right™, USA). The resulting isolates were stored at -20 °C.

4.2.2 Optimization of HVACP Process

4.2.2.1 HVACP Treatment

Protein concentrate was subjected to HVACP treatment through a dielectric barrier discharge (DBD) plasma system (Advanced Plasma Solutions, USA). The main components of the equipment were a reactor chamber with an electrode assembly, a high-voltage transformer, a distribution box, and an exhaust fan. The electrode assembly consisted of an upper high-voltage electrode lined with a dielectric material and a parallelly fixed lower ground electrode. The following processing conditions were maintained consistently during the treatments: frequency of 3500 Hz, the duty cycle of 70%, the pulse width of 10 μ s, the distance from the sample surface to the high-voltage electrode of 2 mm, and the current output of 0–1 A. Treatment voltage (15 kV and 20 kV) and time (1 min, 2 min, 5 min, 10 min, 15 min) were chosen as the variables and 10 treatment combinations were thus developed (**Table 1**).

Table 4.1: Treatment time - voltage combinations and their notations

Treatment	Treatment time				
Voltage	1 min	2 min	5 min	10 min	15 min
15 kV	1T/15V	2T/15V	5T/15V	10T/15V	15T/15V
20kV	1T/20V	2T/20V	5T/20V	10T/20V	15T/20V

4.2.2.2 Carbonyl Content Determination

The carbonyl content of the treated samples was determined using the method outlined by Chanarat et al. (2015). Protein solutions of 20 mg protein/mL were prepared, and a 100 μ L aliquot of each protein solution was reacted with 1 mL of 10 mM 2,4-dinitrophenylhydrazine (DNPH) in 2 N HCl for 1 hrs at room temperature. The control sample was prepared by reacting another 100 μ L aliquot

with 1 mL of 2 N HCl. Following incubation, the solutions were mixed with 1 mL of 20% trichloroacetic acid to precipitate the protein, then centrifuged at 10,000 g for 10 min in a microcentrifuge (Centrifuge 5424, Eppendorf, Germany). The precipitates were then washed twice with 1.5 ml of an ethanol:ethyl acetate (1:1; v/v) mixture to remove unreacted DNPH, and were allowed to blow-dry. Precipitates were then dissolved in 1 mL of 20 mM potassium phosphate (pH 2.3) containing 6 M guanidine hydrochloride. The absorbance at 370 nm was used to measure carbonyl content, and at 280 nm to measure protein content, using a microplate reader. The carbonyl concentration was calculated using a molar extinction coefficient of 22,000 M⁻¹ cm⁻¹.

4.2.2.3 Protein Solubility at pH 7

The protein solubility of the concentrates at pH 7 was determined following the method indicated by Barac et al. (2010), with slight modifications. Protein dispersions (0.5%, w/v) were prepared in Milli-Q water, stirred for 10 minutes, and allowed to hydrate overnight at 4 °C. The hydrated samples were then stirred for 1 hrs, after which the pH was adjusted to 7 using 0.1 M HCl or 0.1 M NaOH. The samples were stirred for an additional 1 hour to enhance protein solubility at the set pH. Following this, the dispersions were centrifuged at 15,000 g for 15 minutes at 22 °C, and the resulting supernatants were collected, measured using a measuring cylinder, and stored at 4 °C for subsequent analysis. The protein content of the supernatant was determined using the Bradford assay, and protein solubility was calculated using the following equation.

$$\text{Protein solubility \%} = \frac{\text{Soluble protein content}}{\text{Protein content in the initial sample}} \times 100$$

4.2.2.4 Selection of optimized conditions

The carbonyl content and protein solubility at pH 7 of each combination were compared statistically. Accordingly, 4 combinations with low carbonyl content and high protein solubility

were chosen for further analysis. Technofunctional properties, compositional and structural characteristics, digestibility, and microbial safety of the treated concentrates were evaluated in comparison to the untreated protein concentrate.

4.2.3 Amino acid analysis

Modified versions of AOAC Methods 982.30 and 994.12, along with ISO 13904:2005(E), were used to determine the content of regular amino acids, oxidized amino acids, and tryptophan, respectively. Quantification of most amino acids was carried out according to AOAC Official Method 982.30 (AOAC, 2012) following 24 hrs acid hydrolysis with 6 N HCl. Methionine and cysteine were determined using AOAC Official Method 985.28, which involved initial oxidation with performic acid, followed by hydrolysis and analysis. Tryptophan was analyzed separately in accordance with ISO 13904:2016 (ISO, 2016). The resulting hydrolysates were derivatized and separated using a UPLC system (Kyoto, Japan) equipped with a Waters AccQ C18 column (100 mm × 2.1 mm, 1.7 µm). Amino acid concentrations were calculated based on hydrated molecular weights, and values expressed as % (w/w) were converted to mg/g protein.

4.2.4 Changes in protein secondary structures

Attenuated total reflectance Fourier transform infrared (ATR–FTIR) spectroscopy was used for structural characterization with an FTIR spectrophotometer (INVENIO S, Bruker Scientific LLC, USA) equipped with OPUS software. Spectra were acquired over the range of 400–4000 cm⁻¹ at a resolution of 4 cm⁻¹, with 120 scans per sample. For each sample, three spectra were collected at room temperature, with a fresh background spectrum (120 scans) recorded prior to each measurement. Powdered samples were placed in direct contact with the ATR diamond crystal, and consistent pressure was applied to ensure measurement uniformity. Spectral processing and

analysis were performed using OriginPro 2025 software (OriginLab Corporation, USA), focusing on the Amide I region (1600–1700 cm^{-1}). After background subtraction, spectra were normalized (0–1), followed by Savitzky–Golay second-derivative processing and peak fitting.

4.2.5 SDS PAGE Analysis

SDS–PAGE was employed to characterize the polypeptide composition of the samples according to the method of Laemmli (1970), with slight modifications (Raikos et al., 2014), using a Mini-Protein electrophoresis unit (Bio-Rad Laboratories Inc., USA). Protein dispersions (4 mg mL^{-1} , w/v) were prepared in 5% sodium dodecyl sulfate (SDS) solution by heating at 85 °C for 1 h, followed by centrifugation at $12,310 \times g$ for 10 min. The final protein samples (2 mg mL^{-1}) were obtained by mixing equal volumes of the protein extract and Laemmli sample buffer. For reducing conditions, 2-mercaptoethanol was added prior to heating the samples at 90 °C for 5 min. Aliquots of 5 μL of each sample, along with 10 μL of Precision Plus Protein™ Standard, were loaded onto a 4–15% Mini-PROTEAN® TGX™ precast gel. Electrophoresis was carried out at 150 V for 1 h using a Mini-PROTEAN II cell. The gels were stained with Coomassie Brilliant Blue R-250 for 1 h and destained for 2 h using a solution containing 50% methanol and 10% acetic acid. Protein bands were subsequently visualized using a ChemiDoc™ imaging system (Bio-Rad Laboratories Inc., USA).

4.2.6 Morphological Characteristics

Scanning electron microscopy (SEM) was used to examine the morphological characteristics of the fine samples using a Quanta 650 FEG microscope (FEI Company, USA) equipped with an Everhart–Thornley (ET) detector. Samples were mounted onto aluminum SEM stubs using double-sided conductive carbon tape and spread as a thin layer prior to imaging. Initially, samples were

sputter-coated with a gold–palladium (Au–Pd) layer using a Desk II sputter coater (Denton Vacuum Inc., USA). It was operated at 50 mTorr and 45 mA for 60 sec. Using the same instrument, the coated samples were subsequently dried under vacuum with nitrogen purging. Micrographs were obtained at magnifications of $\times 500$, $\times 1000$, and $\times 5000$ using xT Microscope Control software (version 6.2.8, FEI Company, USA).

4.2.7 Total Phenolic and Phytic Acid Content

Total phenolic content (TPC) was determined using the Folin–Ciocalteu method as described by Velioglu et al. (1998), with minor modifications. Phenolic compounds were extracted from 200 mg of each sample using 2 mL of 80% methanol acidified with 1% HCl for 2 h at room temperature. Extracts were centrifuged at $2000 \times g$ for 15 min, and a 200 μL aliquot of the supernatant was mixed with 1.5 mL of 10-fold diluted Folin–Ciocalteu reagent and incubated in the dark at 22 °C for 5 min. Subsequently, 1.5 mL of sodium bicarbonate solution (60 g L⁻¹) was added, and the reaction mixtures were incubated in the dark at 22 °C for 90 min. Absorbance was measured at 725 nm using a UV–Visible spectrophotometer (GENESYS 150, Thermo Scientific, USA) against a gallic acid calibration curve. Results were expressed as gallic acid equivalents (GAE). Phytic acid content was determined using the Megazyme Phytic Acid (Phytate)/Total Phosphorus assay kit (K-PHYT, Megazyme International, Ireland) according to the manufacturer's instructions. All analyses were performed in triplicate.

4.2.8 Techno-functional Properties

4.2.8.1 Water Holding Capacity (WHC)

The method specified by Malomo et al. (2014) was used to determine the water-holding capacity of the samples, with modifications. All analyses were conducted in triplicate. Approximately 0.15 g of each sample was weighed into pre-weighed Eppendorf tubes and mixed with 1.5 mL of

distilled water. The mixtures were vortexed for 2 minutes and then allowed to stand for 30 minutes to facilitate water absorption. Subsequently, the samples were centrifuged (Centrifuge 5424, Eppendorf, Germany) at $7000 \times g$ for 25 minutes at room temperature, after which the supernatants were carefully decanted. Excess surface water was drained, and the tubes were reweighed after 15 minutes to determine the amount of water retained by the samples. The water-holding capacity (WHC) was then calculated using the equation provided below.

$$WHC \% = \frac{W2 - (W1 + \text{Sample weight})}{\text{Sample weight}} \times 100$$

Where, W1-empty weight of Eppendorf tube; W2-weight of tube + sample + retained water.

4.2.8.2 Oil Holding Capacity (OHC)

The oil-holding capacity (OHC) of the samples was determined following the same procedure described for WHC in Section 2.3.1, with distilled water replaced by canola oil, as reported by Malomo et al. (2014). The OHC was calculated using the equation provided below.

$$OHC \% = \frac{W2 - (W1 + \text{Sample weight})}{\text{Sample weight}} \times 100$$

Where, W1-empty weight of Eppendorf tube; W2-weight of tube + sample + retained oil.

4.2.8.3 Protein Solubility at Different pH Values

The method outlined by Barac et al. (2010), as mentioned in section 4.2.2.3, was followed to determine protein solubility at pH 3, 5, 7, and 9. Protein solubility was calculated as follows.

$$\text{Protein solubility \%} = \frac{\text{Soluble protein content}}{\text{Protein content in the initial sample}} \times 100$$

4.2.8.4 Emulsifying Activity Index (EAI) and Emulsion Stability Index (ESI)

The analyses were carried out following the method of Karaca et al. (2011) with minor modifications. Buffer systems (0.1 M) used included glycine–HCl (pH 3), sodium acetate (pH 5), sodium phosphate (pH 7), and Tris (pH 9). Protein dispersions (1%, w/v) were prepared and mixed with 5 mL of canola oil, vortexed for 15 sec, and homogenized at 2000 rpm for 1 min (Model 850, Fisher Scientific, Canada). Immediately after homogenization, a 50 µL aliquot of the emulsion was collected from the bottom of the tube, diluted with 5 mL of 0.1% sodium dodecyl sulphate, and vortexed for 10 sec to ensure thorough mixing. The absorbance of the diluted sample was then measured at 500 nm using a UV–Visible spectrophotometer (GENESYS 150, Thermo Scientific, USA). After 10 min, a second 50 µL aliquot was taken from the bottom of the emulsion, diluted in the same manner, and its absorbance was measured. The emulsion activity index (EAI) and emulsion stability index (ESI) were calculated using the equations provided below.

$$EAI (m2/g) = \frac{2 \times 2.303 \times A0 \times N}{C \times \varphi \times 10000}$$

$$ESI (min) = \frac{A0}{\Delta A} \times t$$

Where A0 - absorbance of the diluted emulsion at 0 min; ΔA - differences between the absorbances of the diluted emulsion at 0 min and 10 min; N - dilution factor of the emulsion; C - Protein concentration in g/ml in aqueous phase before emulsion formation; φ - Volume fraction of the oil in total emulsion; t - time interval of emulsion storage.

4.2.8.5 Foaming Capacity (FC) and Foaming Stability (FS)

The analysis was performed following the method of Ajibola & Aluko (2022) with slight modifications. Four 0.1 M buffer systems were used: glycine–HCl (pH 3), sodium acetate (pH 5), sodium phosphate (pH 7), and Tris (pH 9), prepared as described in Section 2.10.4. Protein

dispersions (1%, w/v) were homogenized at 17,000 rpm for 2 min (Model 850, Fisher Scientific, Canada). Immediately after homogenization, the initial foam height was measured using a ruler (0 min). The dispersions were then left to stand for 30 min, after which the remaining foam height was recorded. Foam volume was calculated by converting the measured heights to volume based on the tube dimensions, and foam capacity (FC) and foam stability (FS) were determined using the equations provided below.

$$\text{Foaming capacity} = \frac{V_0}{V} \times 100$$

$$\text{Foaming stability} = \frac{V_{30}}{V_0} \times 100$$

Where, V - volume of the protein dispersion before foaming; V₀ - volume of the foam generated at 0 min; V₃₀ - volume of the remaining foam at 30 min

4.2.9 In Vitro Protein Digestibility and PDCAAS calculation

In Vitro Protein Digestibility (IVPD) values of the samples were determined by using the pH-drop method (AOCS, 2024; Goldberg et al., 2026). Sample solutions were prepared to contain 1 mg N/mL, and their pH was adjusted to 8, and were incubated at 37 °C. A mixture of proteolytic enzymes containing trypsin (1.6 mg/ml), α-chymotrypsin (3.1 mg/mL), and *Streptomyces griseus* peptidase (1.3 mg/mL), was used for the enzymatic digestion. It was added to the protein solution at a 1:10 (v/v) ratio while stirring continuously at 37 °C. The pH change after 10 min (ΔpH) was recorded, and the IVPD was calculated as follows.

$$\text{IVPD} = [74.51 + (22.51 * \Delta\text{pH})]$$

PDCAAS values were calculated following the method prescribed by FAO/WHO (1991). The quantity of each amino acid in each sample was determined as per section 4.2.3, and they were

expressed as mg/ g protein. The division of the indispensable amino acid content of the test protein by the FAO/WHO reference pattern for preschool children's requirements for each amino acid provided the Amino Acid Score (AAS) for each amino acid. The lowest ratio among indispensable amino acids was identified as the limiting amino acid score.

PDCAAS values were obtained by multiplying the amino acid score of the limiting amino acid by the corresponding protein digestibility value, using the equation below.

$$PDCAAS = \text{Amino Acid Score} \times \text{Protein Digestibility}$$

4.2.10 Microbial Inactivation through HVACP treatment

4.2.10.1 Microorganism and Culture Preparation

Assessing microbial inactivation was performed according to the procedure outlined by Los et al. (2018), with some modifications. *Escherichia coli* (ATCC 15597) was used as a surrogate organism to evaluate microbial inactivation by cold plasma treatment. The strain was stored at -80°C in glycerol stocks and revived by streaking onto tryptic soy agar (TSA), then incubated at 37°C for 18–24 hrs. A single colony was transferred to tryptic soy broth (TSB) and incubated at 37°C for 16–18 hrs. The culture's concentration was determined by plate counting. Before inoculation, the bacterial suspension was adjusted to approximately 2.1×10^4 CFU/mL by serial dilution (1:10) in peptone water and confirmed by plate counting.

4.2.10.2 Sample inoculation and cold plasma treatment

Samples of 250 mg from the protein concentrate were aseptically weighed into sterile microcentrifuge tubes. Each sample was inoculated with 1 mL of the prepared *E. coli* suspension (2.1×10^4 CFU/mL), resulting in an initial microbial load of approximately 8.4×10^4 CFU/g. Inoculated samples were gently mixed to ensure uniform distribution under aseptic conditions

before treatment. Inoculated samples were placed between the electrodes and treated at specified voltages and treatment time combinations. Untreated inoculated samples served as controls. Experiments were conducted on two different days, and treatments were conducted in duplicate.

4.2.10.3 Microbial Enumeration and Calculation

Following treatment, the samples were transferred to sterile stomacher bags and thoroughly homogenized. Appropriate serial dilutions (1:10) were prepared, and aliquots were plated onto TSA using the spread plate method. Plates were incubated at 37 °C for 24 hrs, and colonies were counted. Microbial counts were expressed as CFU/g. Microbial reduction was calculated as follows.

$$\text{Population reduction} = (\log \text{population in initial sample}) - (\log \text{population in HVACP treated sample})$$

4.2.11 Statistical Analysis

Data were expressed as the means of replicates. To assess the impact of HVACP treatment on the controls, namely untreated protein concentrate and alkaline-extracted protein isolate, one-way ANOVA followed by Tukey's HSD test was used. The significance level was considered as $p < 0.05$. Statistical analyses are conducted using Minitab 22 Statistical Software (Minitab Inc., USA).

4.4 Results and Discussion

4.4.1 Optimization of HVACP Process

4.4.1.1 Effect of HVACP on protein oxidation

The carbonyl content is an indication of the protein oxidation induced by the HVACP treatment. **Figure 4.1a** shows the carbonyl content for different treatment combinations and indicates an increase in general with increasing time and voltage. Higher carbonyl content was produced at

higher voltage and also increased with time, except for a drop at 10 min. The trends were highly comparable with those recorded in the literature.

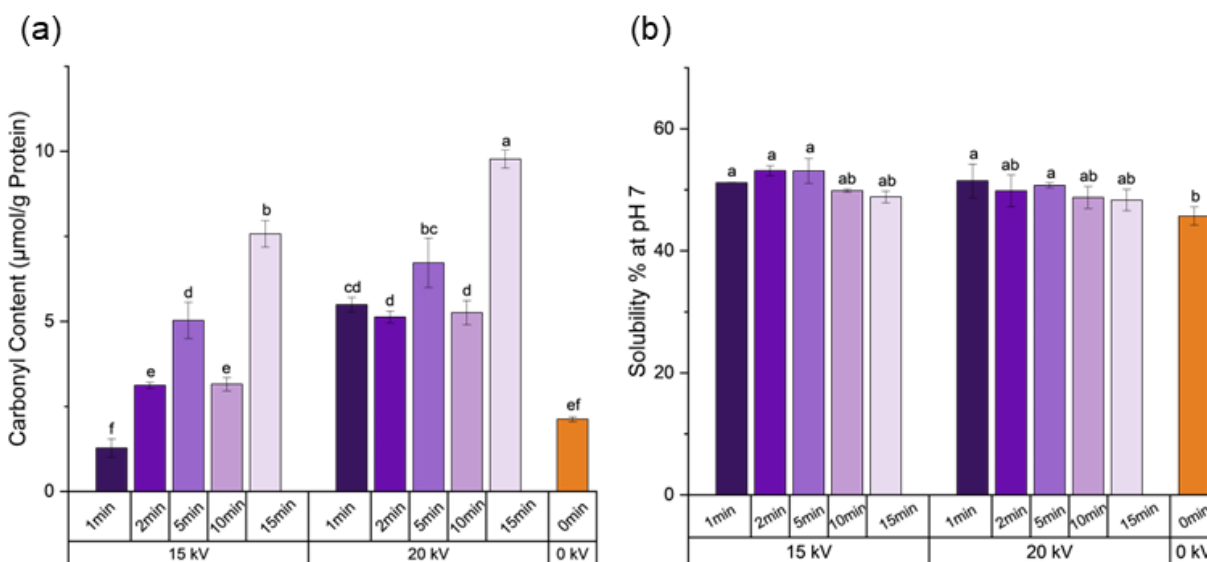


Figure 4.1: Properties of protein concentrates treated at different time-voltage combinations (a) carbonyl content (b) protein solubility at pH 7 (Different lowercase letters show significant differences ($p < 0.05$) - one-way ANOVA ($n=3$))

Formation of carbonyls is associated with oxidative modification of amino acid side-chain residues such as lysine, arginine, proline, and threonine (Olatunde et al., 2023) and is associated with aggregation and polymerization (Luo et al., 2022). Similar trends in the increase in carbonyl content with treatment time were reported by Zhang et al. (2021) for soy protein isolate, Rout & Srivastav (2024) for soy and pea protein isolates, and Chu et al. (2026) for peanut protein isolates. This could be attributed to the effect of treatment time on reactive species concentration, leading to greater protein oxidation, as reflected by increased carbonyl content. Interestingly, Rout & Srivastav (2024) observed a reduction in carbonyl content at 8 min, following an increasing trend up to 6 min, which is in strong agreement with the observations of the current study. In addition,

some studies report a slowing of the rate of increase in carbonyl content over time, which could be due to protein reorganization and newly exposed sites being subjected to ongoing oxidation (Chu et al., 2026).

The impact of higher voltage on increased carbonyl content could be due to the higher energy and density of reactive species, which are enhanced by higher voltage (Mehr & Koocheki, 2020). This increases the likelihood of interactions between reactive species and protein functional groups, leading to higher levels of oxidation. A similar effect of higher voltage was observed for the coconut globulin protein (Chen et al., 2023) and pork myofibrillar proteins (Luo et al., 2022). Accordingly, it was found that carbonyl content increases with treatment intensity and time, indicating unfavorable protein oxidation. Protein oxidation levels are an appropriate parameter for selecting processing parameter combinations when using techniques such as HVACP, which involves reactive species, given the detrimental impacts of oxidation on protein functionality and nutritional outcome (Xiong & Guo, 2020). Therefore, the optimized conditions selected in the current study were based on the minimal increase in carbonyl content relative to the control.

4.4.1.2 Effect of HVACP on protein solubility at pH 7

Figure 4.1b presents the protein solubility at pH 7 recorded for concentrates treated at different treatment combinations. Treatments at 15 kV for 1, 2, and 5 min, and at 20 kV for 1 and 5 min, showed a significant increase in protein solubility, whereas the other combinations remained unaffected. Observations of many studies are consistent in recording that solubility increases initially and decreases as the treatment intensity increases, and are related to protein ingredients such as peanut protein isolates (Ji et al., 2018), soybean protein isolates (Zhang et al., 2021), coconut globulin (Chen et al., 2023), and walnut protein isolates (Deng et al., 2025) among many more. The general trend of increasing solubility can be attributed to HVACP treatment's ability to

dissociate protein aggregates, thereby exposing more active sites and enhancing solubility (Zhang et al., 2021). In addition, it has been reported that particle size and surface charge have a notable impact on protein solubility. HVACP treatments can reduce particle size through aggregate dissociation, and reactive species can also induce additional surface charges, leading to electrostatic repulsion and increased solubility (Mollakhalili-Meybodi et al., 2021). Enhancement of solubility can also be attributed to the introduction of polar groups resulting from the oxidative effect of HVACP treatment (Agarwal et al., 2026). The decrease in protein solubility at higher treatment intensities is often attributed to aggregation and crosslinking induced by reactive species, as well as associated protein denaturation (Hosseini et al., 2025). This could also be due to the protein micelles' crowding effect at the active sites, thereby reducing solubility (Chen et al., 2023). At the same time, oxidative crosslinking induced by the reactive species might also hinder the protein solubility by reducing the available hydrophilic groups, thereby suggesting a nonlinear response of solubility to HVACP treatment (Agarwal et al., 2026). Accordingly, it is clear that the HVACP treatment affects protein solubility, and it is essential to select treatment combinations that increase solubility.

4.4.1.3 Selection of optimized HVACP conditions

Protein carbonyl content and solubility were selected as key parameters for optimizing cold plasma treatment conditions, as they provide complementary insights into protein oxidation and functional performance. Monitoring carbonyl content enables assessment of oxidative damage and helps prevent excessive protein degradation, which could negatively impact the functional properties of the food ingredient. Consuming foods with excess carbonyl content may lead to negative health outcomes, often associated with human health and aging, due to the link between oxidized proteins and diseases such as Alzheimer's disease, chronic aging, Parkinson's disease, cataracts, and

atherosclerosis (Xiong & Guo, 2020). Therefore, the optimized conditions for HVACP treatment must minimize protein oxidation. On the other hand, protein solubility is a critical functional property that directly influences several other functional properties, such as emulsification, foaming, and gelation behavior (Agarwal et al., 2026). Protein solubility also serves as a prerequisite for determining the functional applications of a protein ingredient in different food systems. Therefore, it is important to select HVACP treatment conditions that yield higher protein solubility, as this indicates enhanced functionality.

Accordingly, four treatment time-voltage combinations were selected for further analysis from those evaluated for exhibiting relatively lower carbonyl content and higher protein solubility. Thus, the combinations 1T/15V, 2T/15V, 5T/15V, and 1T/20V were identified as optimal conditions, and their impact on techno-functional properties, compositional and structural characteristics, digestibility, and microbial safety of the treated concentrates was explored and discussed.

4.4.2 Changes in amino acid composition

Table 4.2 indicates the amino acid composition of the treated and untreated protein concentrates. The majority of the amino acids remained unchanged upon HVACP treatment, whereas an increase was observed in Arg, Gly, Phe, and Tyr at 1T/15V treatment. Aromatic amino acids, such as Tyr and Trp, are known to be susceptible to oxidation by reactive species, resulting in a reduction in their concentration (Zhang et al., 2021). However, the increase in Tyr and Phe in the present study was contrary to the above observation, yet aligned with Zhu et al. (2024), who reported a rise in Thr, Phe, and Tyr contents at 10–20 min of treatment for whole-grain flours. Tyr is an aromatic, polar, and partially hydrophobic amino acid, which is detected at the protein surface or within the hydrophobic core of the protein (Moinpour et al., 2020). Similarly, phenylalanine contributes to the hydrophobic core through the aromatic ring (Vugmeyster et al., 2015). Variations in these

amino acids reflect changes in their surrounding molecular environment (Bubler et al., 2015). Evidently, the exposure of the hydrophobic regions of protein molecules due to denaturation caused by the HVACP treatment. Deng et al. (2025) made similar observations and attributed the changes in the Tyr surroundings to depolymerization, chromophore exposure, and loosening of protein structures induced by the protein's interactions with reactive species during HVACP treatment. As hydrophobic interactions between protein molecules are disrupted, surface hydrophobicity increases (Wang et al., 2024), which correlates with enhanced functional properties, such as oil-holding capacity, as evidenced in the current study. Trp is another aromatic amino acid often found in the hydrophobic core of proteins (Bubler et al., 2015), yet such variations were not detected in its concentration. It is the limiting amino acid in lentils as recorded by Osemwota et al. (2022), which agrees with the present study's findings. This could account for the lack of statistically significant variations upon HVACP treatment. Tyr content was significantly reduced between the 1T/15V and 1T/20V treatments, suggesting oxidative degradation as the treatment voltage increased. Zhang et al. (2022) also suggested that sulfur-containing amino acids are highly sensitive to oxidation, yet no significant changes were recorded in the present study. This effect may be attributed to the limiting levels of sulfur-containing amino acids, particularly Met and Cys, which are present at relatively low concentrations in lentil proteins. Based on the observations, it is evident that HVACP treatment directly affects the amino acid composition, thereby altering the techno-functional and structural properties of the protein ingredients.

Table 4.2: Amino acid composition (mg/g protein) of the treated concentrates and control

	1T/15V	2T/15V	1T/20V	PC
HIS	40.07 ± 1.16 ^a	38.78 ± 0.26 ^a	40.29 ± 1.39 ^a	38.51 ± 0.56 ^a

SER	54.10 ± 0.47 ^a	54.41 ± 0.51 ^a	56.54 ± 1.29 ^a	54.31 ± 0.49 ^a
ARG	84.45 ± 0.00 ^a	83.50 ± 0.18a ^b	83.63 ± 1.85a ^b	80.66 ± 0.00 ^b
GLY	40.92 ± 0.33 ^a	38.46 ± 0.02 ^b	40.83 ± 0.90 ^a	40.20 ± 0.44 ^b
ASP	116.67 ± 0.90a ^b	115.81 ± 0.64 ^b	119.71 ± 0.59 ^a	115.93 ± 1.16 ^b
GLU	169.88 ± 0.87a ^b	167.46 ± 0.93 ^b	173.04 ± 1.14 ^a	168.57 ± 1.03 ^b
THR	39.56 ± 0.42 ^a	38.75 ± 0.16 ^a	40.00 ± 0.71 ^a	39.02 ± 0.21 ^a
ALA	43.66 ± 0.04a ^b	42.92 ± 0.16 ^b	44.10 ± 0.39 ^a	43.38 ± 0.06a ^b
PRO	46.61 ± 0.44 ^a	45.47 ± 0.28 ^a	46.92 ± 0.87 ^a	45.67 ± 0.55 ^a
CYS	10.12 ± 0.21 ^a	9.97 ± 0.03 ^a	9.15 ± 0.77 ^a	9.81 ± 0.13 ^a
LYS	74.73 ± 0.35 ^a	73.42 ± 0.16 ^a	76.05 ± 1.02 ^a	74.01 ± 0.85 ^a
TYR	35.62 ± 0.37 ^a	34.26 ± 0.29a ^b	33.72 ± 0.00 ^b	34.08 ± 0.63a ^b
MET	9.28 ± 0.47 ^a	9.55 ± 0.27 ^a	8.36 ± 0.64 ^a	8.89 ± 0.27 ^a
VAL	51.53 ± 0.33 ^a	50.51 ± 0.53 ^a	52.09 ± 1.13 ^a	50.59 ± 0.33 ^a
ILE	46.26 ± 0.08 ^a	45.24 ± 0.47 ^a	47.16 ± 1.26 ^a	45.87 ± 0.38 ^a
LEU	79.62 ± 0.34 ^a	77.87 ± 0.58 ^a	79.05 ± 0.00 ^a	77.99 ± 1.27 ^a
PHE	54.27 ± 0.42 ^b	53.59 ± 0.12b ^c	55.45 ± 0.00 ^a	52.74 ± 0.00 ^c
TRP	8.38 ± 0.15 ^a	8.05 ± 0.05 ^a	8.43 ± 0.16 ^a	8.26 ± 0.21 ^a

(Different lowercase letters show significant differences (p<0.05) - one-way ANOVA (n=3))

4.4.3 Effect of HVACP on protein secondary structures

Figure 4.2 presents a comprehensive overview of the secondary structures identified through FTIR analysis. It provided insights into the molecular-level structural changes in lentil protein concentrates resulting from HVACP treatment, particularly with respect to protein secondary structure. The wide peak observed in the 3000–3600 cm⁻¹ region corresponds to the hydroxyl group

of proteins and starch (Zare et al., 2022). It was observed that the peak intensities of the treated samples were lower than those of the control sample in this region. Changes in the intensity of this region have often been attributed to variations in the moisture content or the number of hydrogen bonds within the structures. Zhu et al. (2024) and Gebremical et al. (2025), in their studies on the effects of HVACP treatment on four different grain flours, made observations similar to those of the present study and attributed these changes to variations in moisture content induced by the HVACP treatment. In relation to the findings of our study, this is highly agreeable, as the treated samples also underwent a freeze-drying step after treatment. In addition, similar observations can also be attributed to the depolymerization of starch glycosidic bonds by the reactive species during the treatment (Chaple et al., 2020). However, changes in this region are highly sensitive to the sample's chemical composition. Zare et al. (2022) reported a decline in hydrogen bonding for quinoa flour, whereas Hosseini et al. (2025) observed an increase in hydrogen bonding for quinoa protein isolate, which has a relatively higher protein content.

Changes in protein upon treatment are mostly characterized by investigating Amide I ($\sim 1600\text{-}1700\text{ cm}^{-1}$) and Amide II ($\sim 1480\text{-}1580\text{ cm}^{-1}$) bands, which correspond to stretching vibrations of the C=O bond and bending vibrations of the N-H bond, respectively (Chaple et al., 2020). A decrease in peak intensity was observed with increasing treatment time, and Gebremical et al. (2025) suggested that this could be due to denaturation caused by the protein's interaction with reactive species. However, several authors reported an increase in peak intensity, attributed to structural unfolding (Sharafodin & Soltanizadeh, 2022) and to changes in the relative proportions of secondary structures, leading to higher stability (Rout & Srivastav, 2024).

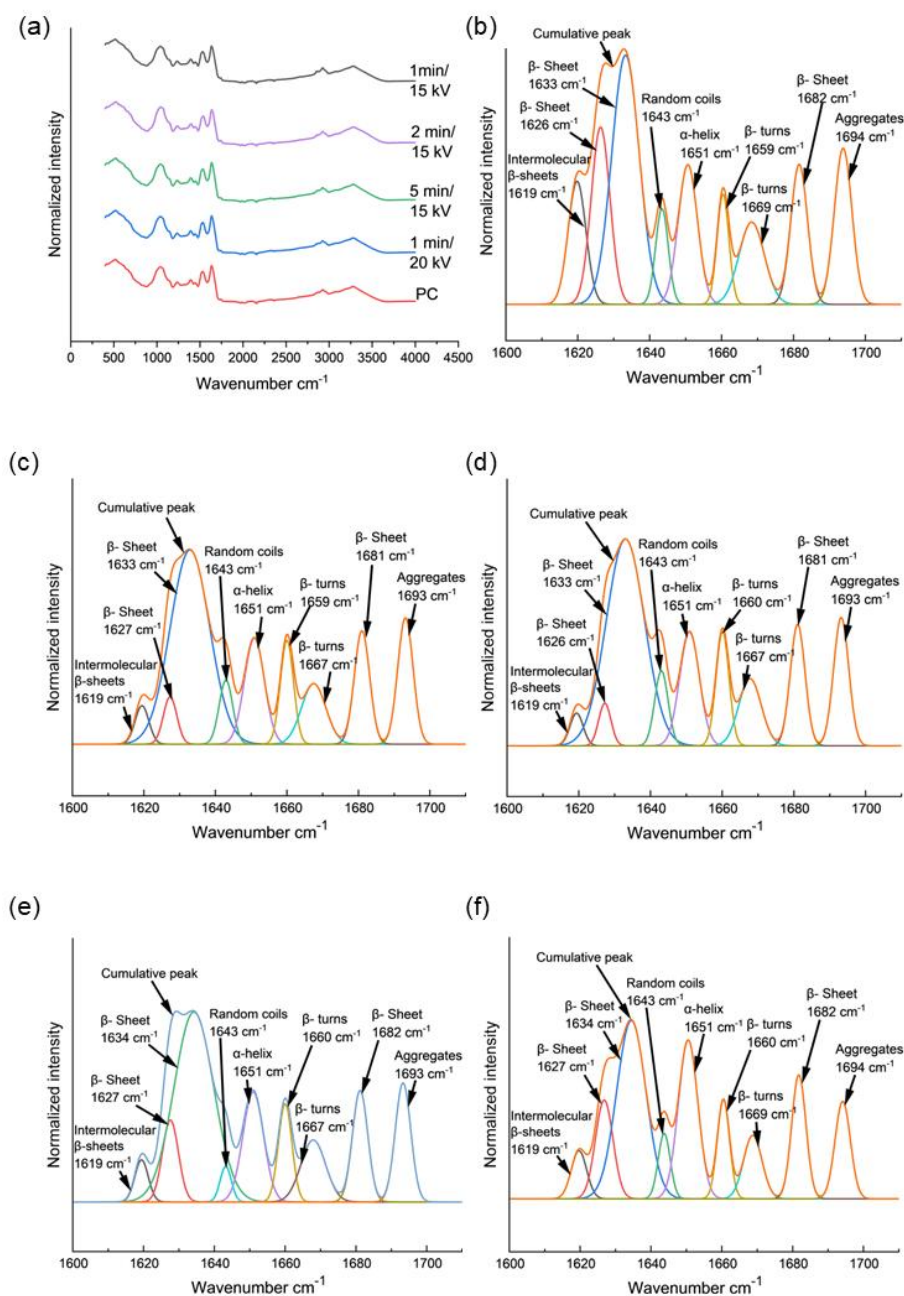


Figure 4.2: Protein structural changes in fine fractions (a) FTIR spectra for HVACP treated and untreated protein concentrates, (b–d) Peak fitting of Amide I with the relative proportion of each secondary structure of (b) 1T/15V (c) 2T/15V (d) 5T/15V (e) 1T/20V (f) Untreated protein concentrate (T-time; V-voltage)

It was expected to observe significant changes in protein secondary structure in samples subjected to HVACP treatment, as reactive species disrupt hydrogen bonding and compromise protein compactness (Yang et al., 2018). Therefore, the Amide I peak was processed using second-order derivatives, and the secondary structures were identified based on their respective wavenumbers (De Angelis et al., 2024). There was a notable decrease in α -helices upon HVACP treatment compared with the control sample. This was also accompanied by a reduction in areas of the β Turns and Intermolecular β -sheet peaks as the treatment intensity increased. This observation suggests a transition from less-ordered to more-ordered secondary structures during HVACP treatment (Mehr & Koocheki, 2020). Most studies on protein isolates report a decrease in α -helices, indicating protein unfolding and structural rearrangements (Wang et al., 2024). In contrast, Deng et al. (2025) observed an increase α -helices following prolonged treatment, attributed to reaggregation of secondary structures, suggesting that HVACP treatment causes aggregation through protein denaturation. The present study's findings also showed that aggregate formation was enhanced by the HVACP treatment compared to the control sample, providing clear evidence of denaturation during HVACP treatment. However, the selected treatments' short exposure times were insufficient to produce a significant difference between treatments (Mehr & Koocheki, 2020). The increasing trend in β -sheet observed in this study is highly consistent with findings from many studies, indicating that HVACP has a prominent effect on protein secondary structure (Wang et al., 2024), ultimately influencing its physicochemical and functional properties.

4.4.4 SDS PAGE Analysis

Figure 4.3 provides the electrophoretic profiles of the treated and untreated samples. SDS-PAGE analysis provided insights into the effects of HVACP treatment on protein primary structure. It was noted that there were no significant changes in the subunit band patterns among the control and treated samples; therefore, the profiles were generally assessed.

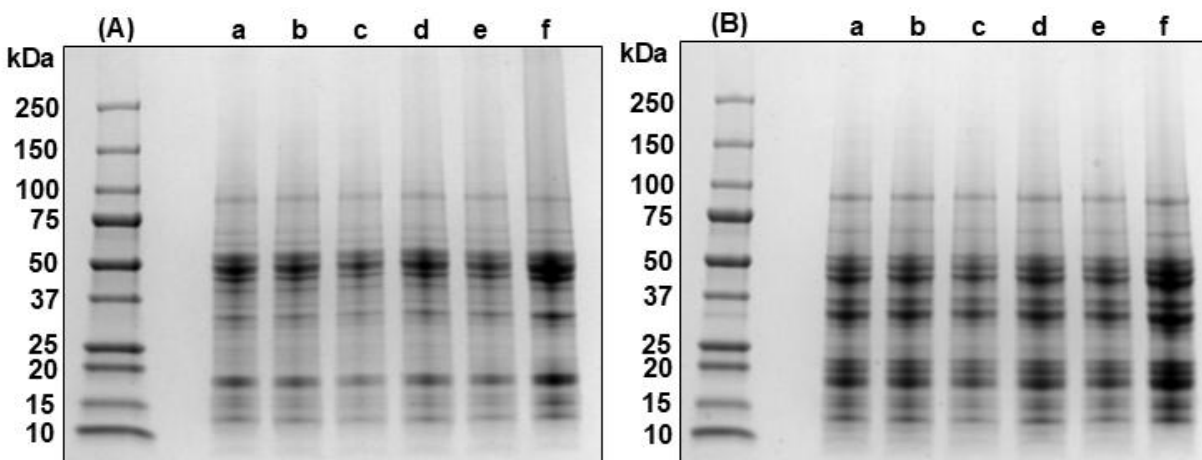


Figure 4.3: SDS-PAGE profile of samples (A) without reducing agent and (B) with reducing agent; Lanes a: 1T/15V; b: 2T/15V; c: 5T/15V; d: 1T/20V; e: PC; f: PI; (T-time; V-voltage; PC-untreated protein concentrate; PI-alkaline extracted protein isolate)

Under non-reducing conditions, protein bands were observed at approximately 64, 48, 42, 32, and 16–19 kDa. Bands detected in the 15–19 kDa range are associated with γ -vicilin and with polypeptides generated by cleavage of storage proteins (Ghumman et al., 2016). Bands appearing around 60–64 kDa are attributed to legumin subunits, which assemble via non-covalent interactions to form the 11S legumin protein, a hexameric structure with a molecular weight of approximately 380 kDa (Miranda et al., 2022). Alonso-Miravalles et al. (2020) assigned bands near 50 kDa to vicilin, which may explain the pronounced bands observed at approximately 48

kDa in the present study. Vicilin is a major globulin characterized as a 7S trimeric protein with an overall molecular weight of about 150 kDa. Similarly, Barbana & Boye (2013) reported vicilin-related bands in this region and attributed bands around 64 kDa to convicilin.

Under reducing conditions, the partial disappearance of bands at 60–64 kDa was observed, likely due to the cleavage of disulfide bonds separating the acidic α -chain (~40 kDa) and basic β -chains (~20 kDa) of legumin (Miranda et al., 2022). Concurrently, increases in band intensity at approximately 37 kDa and 20 kDa further confirmed the formation of acidic and basic chains, respectively. Comparable banding patterns under reducing conditions, including a reduction in the intensity of bands near 50 kDa, have been reported by Alonso-Miravalles et al. (2020), consistent with the observations of the present study. The formation of acidic polypeptides under reducing conditions was further evidenced by bands at 32, 42, and 47 kDa (Jarpa-Parra et al., 2015).

According to the current study's observations, there were no significant differences in the band patterns of the samples, indicating that the HVACP treatment did not alter the covalent and non-covalent interactions of the proteins. This was a common observation across many studies, including peanut protein isolates (Ji et al., 2018), pea protein isolate (Rout & Srivastav, 2024; Liu et al., 2024), and sunflower seed protein (P. Wang et al., 2024), regardless of treatment voltage or intensity. However, some studies on animal proteins indicate that HVACP treatment was effective in altering protein composition, unlike in plant proteins. Luo et al. (2020) documented a disappearance of myosin heavy chain at higher treatment intensities, suggesting that reactive species led to protein aggregation. Cheng et al. (2023) also observed that the bands in shrimp tropomyosin profiles shifted upward, with an increase in molecular weight, evidently due to partial covalent crosslinking induced by plasma oxidation. In contrast, such crosslinking or aggregation

was not observed in the casein profiles (Zhang et al., 2024), indicating that the effect of HVACP treatment on changes in primary structure is highly dependent on the protein's characteristics.

4.4.5 Morphological Characteristics

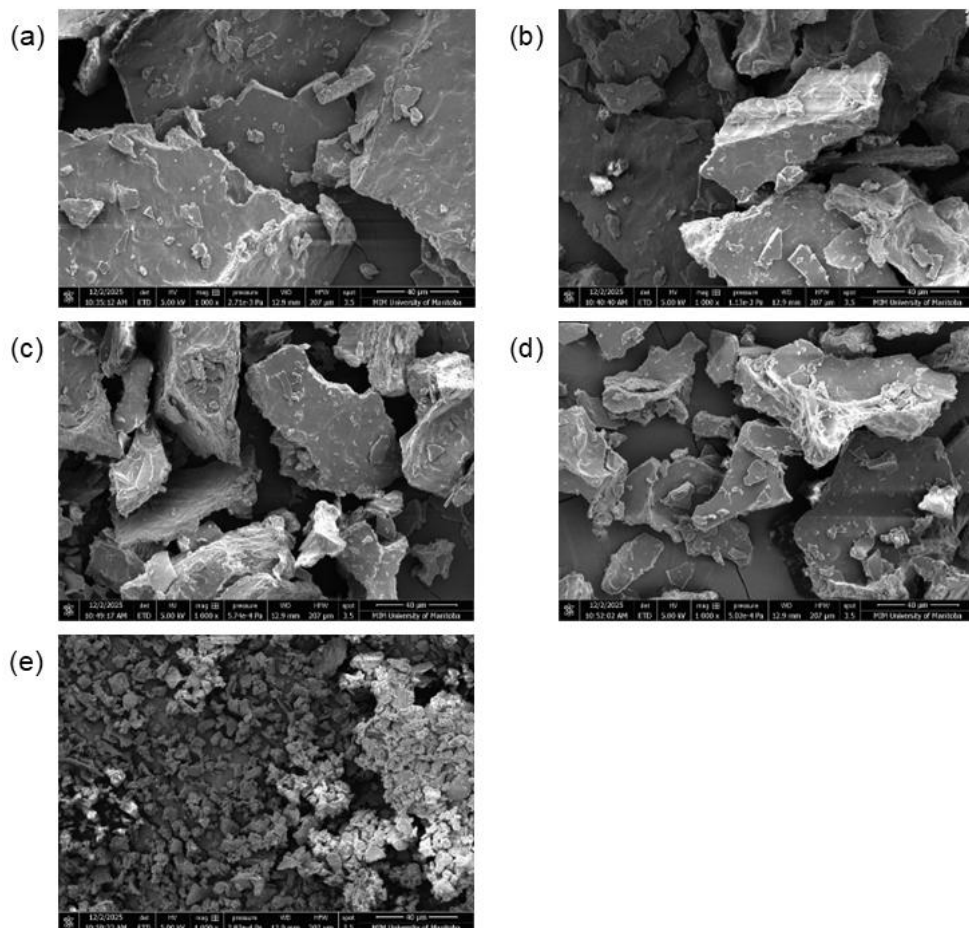


Figure 4.4: Morphological characteristics of (a) 1T/15V; (b) 2T/15V; (c) 5T/15V; (d) 1T/20V € PC (amplification factor – x 1000) (T-time; V-voltage; PC-untreated protein concentrate)

Scanning electron micrographs of the samples are presented in **Figure 4.4** and were used to examine the surface morphology and microstructural changes of lentil protein concentrates following HVACP treatment. The HVACP-treated samples exhibit a sheet-like morphology, which is most likely conferred by the freeze-drying process that followed HVACP treatment (Basyigit et al., 2022). It was noted that as treatment intensity increased, the sheet-like structures became

smaller, giving a more fragmented appearance. Liu et al. (2024) made a similar observation with pea protein isolates, attributing this reduction to protein unfolding and an increase in surface hydrophobicity. This was also true for globular soy protein isolates, as observed by Sharafodin & Soltanizadeh (2022), who reported a more orderly, more distanced arrangement of the protein globules upon HVACP treatment. This was attributed to repulsive forces arising from charged functional groups on the protein surface as they interact with the reactive species during the treatment. The movement to loosen particles from the dense, compact structure of the control upon HVACP treatment was also commonly observed in walnut protein isolates (Deng et al., 2025) and sunflower seed proteins (Wang et al., 2024).

Changes in surface roughness are another major observation reported by many authors in this regard. In the present study, it was observed that the protein's surface roughness decreased with increasing treatment intensity, as the surface appeared smoother and more homogeneous, with fewer wrinkles and deformations. These changes in the surface could be caused by surface etching, a phenomenon involving bond-breaking on the protein surface as proteins are exposed to high-energy particles and undergo rapid oxidation during the HVACP treatment (Sharafodin & Soltanizadeh, 2022). In some cases, surface roughness is reported to be enhanced by the treatment, as high-energy particles collide with the surface and initiate chemical reactions involving bond and chain breakage (Liu et al., 2024). Surface etching also leads to the formation of surface holes, which become more abundant at longer treatment durations due to prolonged exposure of proteins to high-energy particles (Sharafodin & Soltanizadeh, 2022). This was observed in the current study as well, indicating that the same phenomenon affected the surface morphology and may have impacted other structural and functional properties of the treated concentrates. Surface etching can be classified into two types of reactions: physical reactions due to collisions between the protein

surface and reactive species, or chemical reactions that result in the formation of volatile gaseous products and radicals by affecting the protein structure (Agarwal et al., 2026). Similarly, it can cause the formation of cavities or holes on the surface at higher treatment intensities, as observed in the current study and in soy protein isolates (Sharafodin & Soltanizadeh, 2022). More holes were recorded with increased duration, due to greater exposure to reactive species, resulting in a significant effect on the protein.

4.4.6 Total Phenolic and Phytic Acid Content

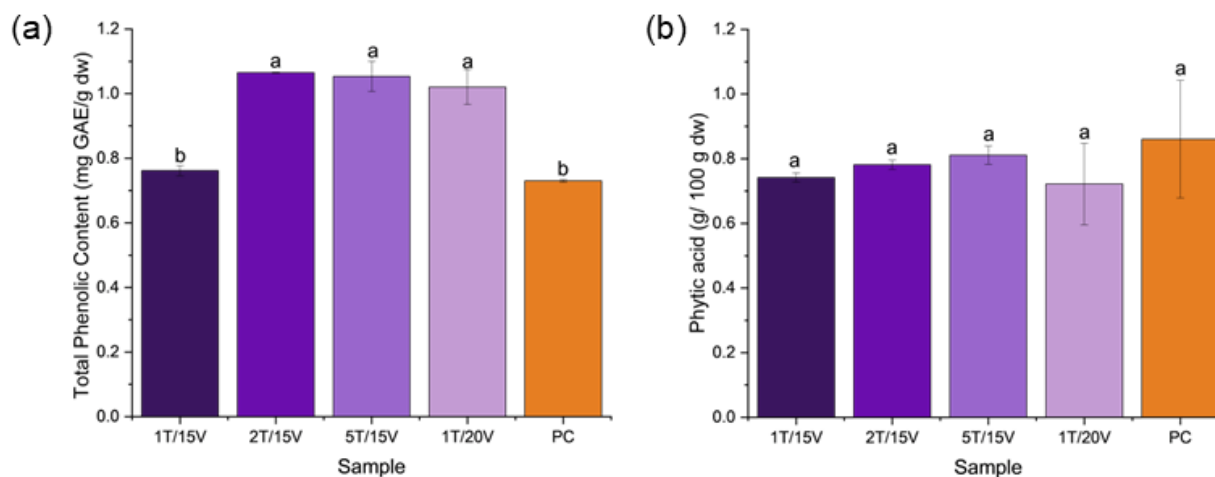


Figure 4.5: (a) Total phenolic content; (b) Phytic acid of HVACP treated and untreated protein concentrates (Different lowercase letters show significant differences ($p < 0.05$) - one-way ANOVA (phytochemicals $n=3$) (T-time; V-voltage; PC-untreated protein concentrate; PI-alkaline extracted protein isolate)

Figure 4.5 depicts the effects of HVACP treatment on the total phenolic and phytic acid contents of protein concentrates. Polyphenols are often regarded as bioactive compounds, yet they may also act as antinutritional factors owing to their propensity to bind proteins via sulfhydryl groups and free amino acids (Rout & Srivastav, 2024). **Figure 4.5a** shows the total phenolic content of the

samples and indicates a significant increase with higher treatment intensity. The increase was not significant at 1T/15V, yet was highly significant in all other treatments.

Delving into the results from past studies, a consistent pattern emerges: the polyphenolic content increases with mild cold plasma treatment and may degrade with high-intensity treatments. These increments showed a voltage and time-dependent manner. Rout & Srivastav (2024) noted an increase in the phenolic content in soy protein isolate at mild treatment time-voltage combinations, which reduced as the treatment intensified. In buckwheat, two flavonoid compounds, namely rutin and quercetin, were significantly enhanced upon cold plasma treatment, whereas the total and phenolic and flavonoid contents decreased with the highest treatment condition (M. Amiri et al., 2025). In contrast, Jamali et al. (2025) didn't observe any decline in the contents within the tested treatment conditions. Interestingly, several enzyme activities, including phenylalanine ammonia-lyase (PAL), were reported to be enhanced in blueberries, which play an important role in the biosynthesis of phenolic acids, flavonoids, and anthocyanins (Zhou et al., 2023). Thus, it is clear that the cold plasma treatment has a positive influence on the release of pre-existing phenolic compounds and on the biochemical induction of new phenolics.

To provide mechanistic insights into the above observations, all authors attributed the increase in polyphenolic compounds to several phenomena induced by the cold plasma treatment. A primary attribution was the enhancement of phenylalanine ammonia-lyase (PAL) activity, thereby stimulating the conversion of phenylalanine to phenolic acids (Zhou et al., 2023). The extractability of phenolic compounds is also expected to increase upon cold plasma treatment, as reactive species degrade cell membranes, thereby enhancing the recorded polyphenolic content (M. Amiri et al., 2025). Another proposed mechanism is the fragmentation of larger polyphenolic compounds into smaller, measurable molecules, thereby enhancing their detectability (Jamali et

al., 2025). However, the reduction in polyphenol content under high-intensity treatments can be explained by their antioxidant activity, as reactive oxygen species and ozone can attack the aromatic rings of polyphenols, converting them into aliphatic compounds, which subsequently rupture and degrade (M. Amiri et al., 2025). Some studies have effectively used cold plasma treatment to reduce phenolic compounds in some samples, leveraging this phenomenon (Wang et al., 2020).

According to **Figure 4.5b**, the phytic acid content of the treated samples decreased; however, no significant difference was observed compared with the control. Arjmand et al. (2025) reported a decrease in phytic acid content following HVACP treatment, attributed to reactive species breaking the phytate ring and causing structural damage to phytic acid. HVACP treatment also enhances various enzymes, such as phytase, which could contribute to this reduction in phytic acid content (Sruthi et al., 2022). A decrease in phytic acid was also observed in guava seed flour, as reported by Kheto et al. (2023), who noted that greater reductions are possible with prolonged treatment durations. This was attributed to the fact that extended treatment time accelerates its effect on the phytate ring, leading to greater structural destruction. Similar observations were made by Patra et al. (2024) for horse gram flour and Debjani Das et al. (2024) for groundnut. Despite extensive study of whole-seed flours, corresponding findings for protein ingredients are limited and warrant further exploration. However, the role of HVACP treatment in reducing phytic acid content can be appreciated, and the technology can be effectively utilized to overcome antinutritional and digestive problems associated with plant protein ingredients.

4.4.7 Effect of HVACP on Techno-functional Properties of Protein Concentrates

4.4.7.1 Water Holding Capacity (WHC)

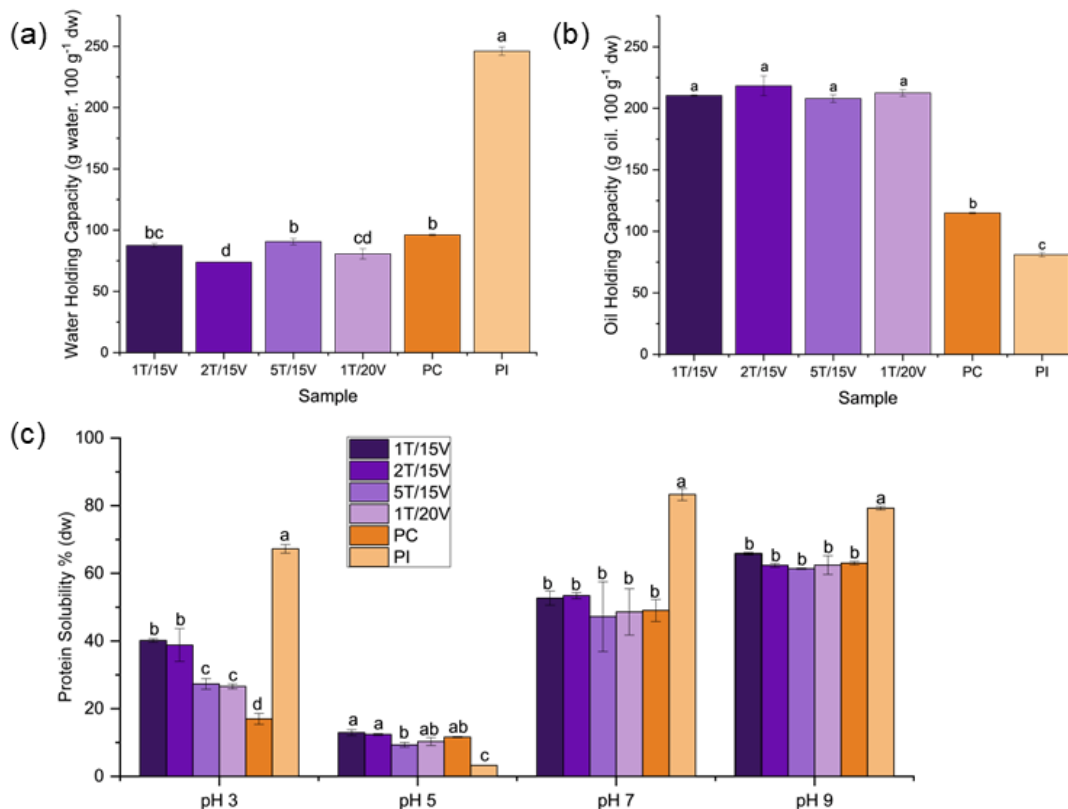


Figure 4.6: Techno-functional Properties of fine fractions compared with controls (a) Water holding capacity (b) Oil holding capacity (c) Protein solubility (Different lowercase letters show significant differences ($p < 0.05$) - one-way ANOVA ($n=3$)) (T-time; V-voltage; PC-untreated protein concentrate; PI-alkaline extracted protein isolate)

Figure 4.6a shows the WHC values of the treated samples, which decrease compared to those of the untreated sample. WHC is significantly reduced at 2T/15V and 1T/20V, where it remains unchanged in other two treatment combinations. It is understood that observations reported in the literature on the impact of HVACP treatment on WHC are highly dependent on the material under

consideration. A significant decline in the WHC was recorded with quinoa flour, which was identified to be due to the reduction of the exposed hydroxyl groups as depicted by the FTIR spectra at the region ($3000\text{-}3600\text{ cm}^{-1}$), thus causing lower WHC (Zare et al., 2022). However, an increase in WHC was recorded for several other flours, including parboiled rice (Sarangapani et al., 2016).

Findings on WHC of protein isolates show a similar trend. Rout & Srivastav (2024), Sharafodin & Soltanizadeh (2022), and Liu et al. (2024) reported an initial increase in WHC for soy protein isolates and pea protein isolates, followed by a decrease as the treatment intensity increased. The increase in the hydrophilicity of these proteins was attributed to protein oxidation and amino acid ionizations, a response to the highly reactive species generated during HVACP treatment (Rout & Srivastav, 2024). At the same time, protein unfolding enhances interactions between proteins and water, initially increasing WHC, which is later restrained at higher treatment intensities due to protein aggregation, leading to a larger particle size (Liu et al., 2024). The enhanced WHC could also be due to surface etching caused by the cold plasma treatment, which modifies physical properties and protein interaction (Rout & Srivastav, 2024). Sharafodin & Soltanizadeh (2022) also suggested that carbonyl group formation due to protein oxidation could be a contributing factor, as it encourages more hydrogen bond formation.

4.4.7.2 Oil Holding Capacity (OHC)

The OHC values of the treated concentrates are shown in Figure **4.6b** and show a significant increase compared to the control samples. This suggests a notable increase in the hydrophobicity upon HVACP treatment. This is highly consistent with the observations recorded in the literature. The surface hydrophobicity is expected to increase with HVACP treatment, as nonpolar amino acids and hydrophobic groups become more exposed and form stronger interactions with oil,

leading to more protein-oil complexes (Liu et al., 2024). A similar explanation was reported by Hosseini et al. (2025) for observations similar to those in quinoa protein isolates. This observation could also be correlated with the reduction of hydrophilic groups in protein and starch, as further evident by the reduced -OH tensile vibration in the FTIR spectra (Zare et al., 2022), as discussed in section 3.2.1. Accordingly, the increase in OHC observed in the present study can also be attributed to the reduction in hydrophilic groups, as shown in the FTIR spectra (**Figure 4.2**). In addition, amino acid analysis of the treated samples shows an increase in hydrophobic amino acids, such as Tyrosine and Phenylalanine (Table 4.2), which hints at enhanced exposure of hydrophobic groups due to the HVACP treatment, contributing to increased OHC.

Most studies have documented a decrease in OHC with increasing treatment intensity, and several explanations have been proposed to account for this phenomenon. At 60 kV, a reduction in OHC was observed in quinoa protein isolates compared with treatment at 50 kV, likely due to HVACP-induced conformational rearrangements that exposed hydrophilic groups previously buried within the protein matrix (Hosseini et al., 2025). At the same time, re-aggregation could also reduce OHC as the exposure of binding sites becomes limited (Deng et al., 2025). This was not apparent in the present study, which may be because the optimized conditions are mild and capable of preventing protein aggregation. Significant changes in OHC were not prompted by HVACP treatment in wheat flour (Chaple et al., 2020).

4.4.7.3 Protein Solubility at Different pH Values

Based on the results of this study (Figure 4.6c), protein solubility increased significantly at pH 3, whereas no significant differences were observed at neutral and alkaline pH levels. This trend may be associated with potential structural and compositional changes in the protein caused by HVACP

treatment. Generally, HVACP treatment is expected to have a positive effect on protein solubility for several reasons, as discussed in section 4.4.1.2.

Furthermore, it is worth discussing why significant differences were observed only at acidic pH, but not at neutral or alkaline pH. Under alkaline conditions, the loss of activity in proteins and peptides is mainly due to structural modifications in asparagines and glutamines, which are highly sensitive (Gülich et al., 2000). However, the amino acid analysis indicated that these amino acids are present in high abundance in lentil protein concentrates, with no significant changes in their concentrations following HVACP treatment (**Table 4.2**). This could explain why no significant change in solubility was observed at pH 9. The negative charge imparted on the protein molecules by the alkaline conditions maintains electrostatic repulsion between them, keeping them well dispersed and resulting in higher solubility at pH 9 (Ngui et al., 2021). Therefore, it could also be stipulated that, since the concentrates already had higher solubility at alkaline pH values, HVACP treatment was unable to induce structural or conformational changes that would significantly alter solubility at the respective pH. A relatively higher solubility at pH 7 could also be ascribed to a similar negative charge distribution on the protein surface as the surrounding pH moves away from the isoelectric pH. Similar to pH 9, this could lead to the absence of significant changes in protein solubility at pH 7.

The reduced protein solubility observed at pH 5 can be attributed to its proximity to the isoelectric point (pI) of lentil proteins, where electrostatic repulsion between protein molecules is minimized, thereby promoting protein–protein interactions and aggregation (Miranda et al., 2022). Globulins, which constitute the predominant protein fraction in lentils, exhibit a pI near pH 5 and therefore tend to precipitate under these conditions (J. Yang et al., 2023). Consequently, the soluble protein

fraction at this pH is primarily composed of albumins, which are present at lower abundance, resulting in a reduction in overall protein solubility.

Increased solubility at pH 3 is mediated by the occurrence of a positive charge on the protein surface at acidic conditions (Ngui et al., 2021). Despite no significant change in other pH values in the current study, protein solubility was significantly increased at pH 3. At acidic pH, the exposure of protonatable amino acid residues, such as amino and carboxyl groups, increases the surface positive charge, reducing protein–protein aggregation and promoting protein–water interactions (Schönichen et al., 2013). Protein oxidation, which is apparent following HVACP treatment, could thus enhance protein solubility at pH 3 by exposing carbonyl groups and can be identified as a contributing factor to the observed effects. At the same time, there is a significant decrease in protein solubility at pH 3 as the treatment intensity increases, which could be due to crosslinking-induced aggregation during HVACP treatment (Hosseini et al., 2025). However, it is evident that protein solubility at acidic pH is significantly affected by HVACP treatment.

The number of studies investigating the effects of HVACP treatment on protein solubility across different pH values remains limited, restricting a comprehensive understanding of the underlying mechanisms. Consequently, this area requires further scientific investigation to fully elucidate the potential of HVACP in enhancing protein functionality.

4.4.7.4 Emulsifying Activity Index (EAI) and Emulsion Stability Index (ESI)

Figure 4.7a and 4.7b indicate the impact of HVACP treatment on the emulsifying properties of the concentrates. Observations show that HVACP-treated proteins can orient at the oil droplet surface, thereby stabilizing the emulsion through hydrophobic and hydrophilic interactions. Based on the observations of the present study, HVACP treatment did not adversely affect the emulsifying properties of lentil protein concentrates, except for a reduction in emulsion stability at pH 3. It

shows an increase compared to the control at pH 3, but a decreasing trend with increasing treatment intensity. Similar observations of an initially increasing and then decreasing trend were made by Hosseini et al. (2025) for quinoa protein isolates, Chen et al. (2023) for coconut globulin, Q. Zhang et al. (2021) for soy protein isolates, and Rout & Srivastav (2024) for pea and soy protein isolates.

Several mechanisms can explain the enhancing effect of HVACP on emulsifying properties. One such frequently suggested mechanism is that reaction with reactive species breaks intermolecular bonds within proteins, leading to changes in protein conformation at the oil-water interface.

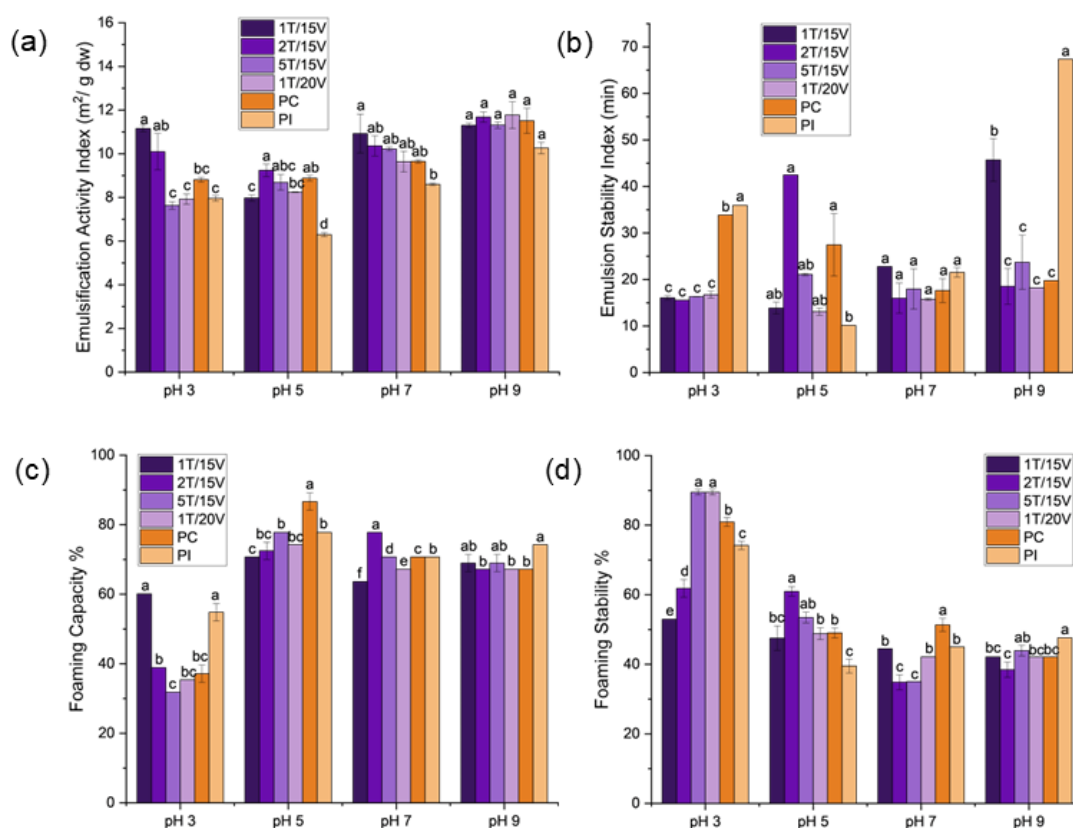


Figure 4.7: Techno-functional Properties of fine fractions compared with controls (a) Emulsifying activity index (b) Emulsifying stability index (c) Foaming capacity (d) Foaming stability (Different lowercase letters show significant differences (p<0.05) - one-way ANOVA (n=3)) (T-time; V-voltage; PC-untreated protein concentrate; PI-alkaline extracted protein isolate)

Accordingly, the protein molecules become more flexible, and the interfacial tension is reduced, thus favoring the formation of emulsions (Deng et al., 2025). At higher treatment intensities, emulsifying properties decline, possibly due to aggregation that restrains interfacial characteristics (Zhang et al., 2021). It can also be noted that the hydrophilicity of proteins is enhanced with HVACP treatment, as evidenced by its impact on protein solubility. This supports the arrangement of proteins at the water-oil interface, thereby improving EAI (Zhang et al., 2020). In addition, HVACP's effect on particle size also affects emulsifying properties. Chen et al. (2023) observed a correlation between decreasing particle size and increasing intensity of HVACP treatment, leading to a more homogenized distribution of oil droplets and better emulsion stability. It is noteworthy that a significant increase in EAI for treatment 1T/15V at pH 3 was observed, which correlates with the increased solubility of the treated samples at pH 3. Emulsifying activity index is strongly influenced by protein solubility, as higher solubility facilitates protein diffusion and adsorption at the oil–water interface (Chen et al., 2023). However, trends related to EAI and ESI differ based on several factors related to the protein source, such as their 7s and 11s globulin composition, amino acid composition, carbohydrate content, etc. (Yang et al., 2021).

4.4.7.5 Foaming Capacity (FC) and Foaming Stability (FS)

Figure 4.7c and 4.7d indicate that the foaming characteristics of the protein concentrates were seemingly enhanced upon the HVACP treatment. At pH 3 and 7, FC was significantly increased at milder treatments, then decreased as the treatment duration and voltage increased. The increase in foaming capacity (FC) could be correlated with enhanced protein solubility, as observed for the EAI. Similar to ESI, FS was significantly decreased at pH 3.

Most prior studies report an initial increase in FC, followed by a reduction with increasing treatment duration, as is commonly observed for the other techno-functional properties discussed

above. This is also true for the current study, although the differences are not significant in certain pH conditions. FC values also showed a strong correlation with protein solubility values, indicating the crucial role of protein solubility in determining foaming capacity (Hosseini et al., 2025). Higher protein solubility offers more protein molecules to participate in foaming, thereby increasing the FC (Chen et al., 2023). The increased foaming capacity could result from protein unfolding during the HVACP treatment, leading to greater flexibility at the water-air interface and thus forming more foam (Rout & Srivastav, 2024). Another contributing factor is the etching effect of HVACP treatment, which dissociates protein aggregates, thereby exposing more hydrophobic groups and increasing surface activity (Bußler et al., 2015). HVACP treatment also increases surface charge, promoting greater interactions at the water-air interface and reducing surface tension (Zhang et al., 2020). It was also noted that the ESI decreased upon HVACP treatment, especially at pH 7, which could be attributed to a weak surface layer around the bubbles, resulting from loss of secondary structure rigidity and protein conformational stability (Agarwal et al., 2026).

4.4.8 Effect of HVACP on In Vitro Protein Digestibility and PDCAAS

The number of studies assessing the impact of HVACP on the in vitro protein digestibility (IVPD) of protein ingredients is highly limited; hence, a notable research gap exists in this regard (Olatunde et al., 2023). As indicated in **Table 4.3**, in the current study, the IVPD values were significantly increased after HVACP treatment compared to the untreated sample. Similar observations were recorded in relation to soy and pea protein isolates, with soy protein isolates showing better IVPD compared to pea protein isolates. The authors attributed this difference to the higher trypsin-inhibitory activity in soy protein isolates (Rout & Srivastav, 2024). Enhancing digestibility by HVACP could be due to the conformational changes induced by the reactive species, such as unfolding and exposing the active sites for more proteolytic enzymes, facilitating

the digestion process (Olatunde et al., 2023). Kheto et al. (2023) recorded an increase in IVPD with HVACP treatment duration, which was attributed to the reduction of the antinutritional factors in guava seed flour. Furthermore, HVACP treatment has a profound effect on the amino acid profiles of the protein samples, especially in sulfur-containing amino acids, which in turn affects the level of crosslinking within protein molecules. This results in the breakdown of proteins further facilitating the digestion process.

Table 4.3: Digestibility and PDCAAS values of protein concentrates and controls

	1T/15V	2T/15V	1T/20V	PC
LEAA^a	TRP	TRP	MET + CYS	MET + CYS
IVPD^b	95.33 ^a	95.22 ^{ab}	95.44 ^a	94.54 ^b
PDCAAS^c	72.63	69.68	66.89	70.72
Total EAA	449.45 ± 3.57 ^a	439.97 ± 0.63 ^a	449.74 ± 5.22 ^a	439.79 ± 1.79 ^a
Total NEAA	556.29 ± 0.56 ^{ab}	548.03 ± 1.75 ^b	564.77 ± 7.02 ^a	548.73 ± 1.75 ^b
Total AA	520.49 ± 1.56 ^a	514.07 ± 0.58 ^a	518.34 ± 6.25 ^a	510.72 ± 0.02 ^a

(T-time; V-voltage; PC-untreated protein concentrate; ^aLimiting essential amino acid; ^bIVPD = In vitro protein digestibility; ^cPDCAAS = AAS*IVPD (Different lowercase letters show significant differences (p<0.05) - one-way ANOVA (n=3))

Plant proteins are known to possess inherently compact and ordered structures, stabilized by extensive hydrogen bonding, hydrophobic interactions, and disulfide linkages, which can restrict the accessibility of digestive enzymes. In addition, plant protein matrices are often associated with antinutritional factors such as phytic acid, tannins, and trypsin inhibitors, further limiting

enzymatic hydrolysis during gastrointestinal digestion (Sá et al., 2020). These structural constraints and antinutritional barriers collectively contribute to the lower digestibility of plant proteins compared to animal proteins. On the other hand, HVACP treatment has proven its potential in disintegrating complex protein structures and lowering the abundance of antinutritional factors in protein ingredients, thereby being identified as an effective strategy to enhance the digestibility and nutritional quality of these ingredients.

Table 4.3 also presents the calculated PDCAAS values. Accordingly, all samples recorded PDCAAS values within the range 66.89-72.63, indicating moderate-quality proteins. Most Canadian pulses have PDCAAS values around 0.5, indicating that the lentil protein ingredients studied in the present study have relatively better nutritional properties (Nosworthy et al., 2017b). The detailed calculation of PDCAAS values is indicated in **Supplementary Material 2: Amino acid composition and PDCAAS calculation of HVACP treated and untreated samples**.

It was also noted that the PDCAAS values decreased with intensifying HVACP treatment, accompanied by a change in the limiting amino acid. At lower treatment intensities, Tryptophan acted as the limiting amino acid, but was replaced by sulphur-containing amino acids as the treatment voltage increased. As discussed in section 4.4.2, Tryptophan is an aromatic amino acid present in the hydrophobic core of a protein molecule (Bußler et al., 2015), which is expected to increase in quantity during HVACP treatment due to denaturation and unfolding induced by reactive species. It can be suggested that this caused an increase in tryptophan content, preventing it from being identified as the limiting amino acid in the 1T/20V-treated concentrates. On the other hand, this could be affected by a simultaneous decrease in sulfur-containing amino acids, as they aggregate. Oxidation of sulphur-containing amino acids due to exposure to reactive species can induce the formation of disulphide bonds and sulphur crosslinks, resulting in protein aggregate

formation (C.-H. Tang et al., 2009). The FTIR diagrams (**Figure 4.2**) also indicate higher aggregate formation at the higher-intensity treatments, which could support the above stipulation. It is also known that cold plasma treatment causes disintegration of protein aggregates at lower-intensity treatments, which, in turn, favors aggregation at higher-intensity treatments (Hosseini et al., 2025). Therefore, changes in the limiting amino acid can be closely associated with changes in protein structure, thereby providing insight into their impact on PDCAAS values.

The relationship between HVACP treatment and protein digestibility-related aspects remains largely underexplored, and to the best of our knowledge, the current study is the first to calculate PDCAAS values for HVACP-treated samples. Therefore, further studies are needed to provide detailed explanations for the trends observed with HVACP treatment and the digestibility of the protein ingredients.

4.4.9 Microbial Inactivation through HVACP treatment

The present study investigated the dual function of HVACP to establish the optimal treatment conditions, while bridging an important research gap. **Figure 4.8** demonstrates the positive effect of treatment on microbial population reduction, especially when the treatment voltage was increased rather than the treatment duration. A significant reduction, approximately 2.0 log units, was achieved with the treatment combination 1T/20V, corresponding to a brief exposure at a higher voltage. A similar log reduction was observed in association with peanut protein by Chu et al. (2026), further supporting the idea of HVACP's dual functionality. Assessing the impact of HVACP on a supplement powder, H. Amiri et al. (2025) also reported a complementary effect of the treatment in enhancing its microbial safety and sensorial properties. They achieved ~2.5 log reductions in bacteria, depending on the type, with extended treatment times.

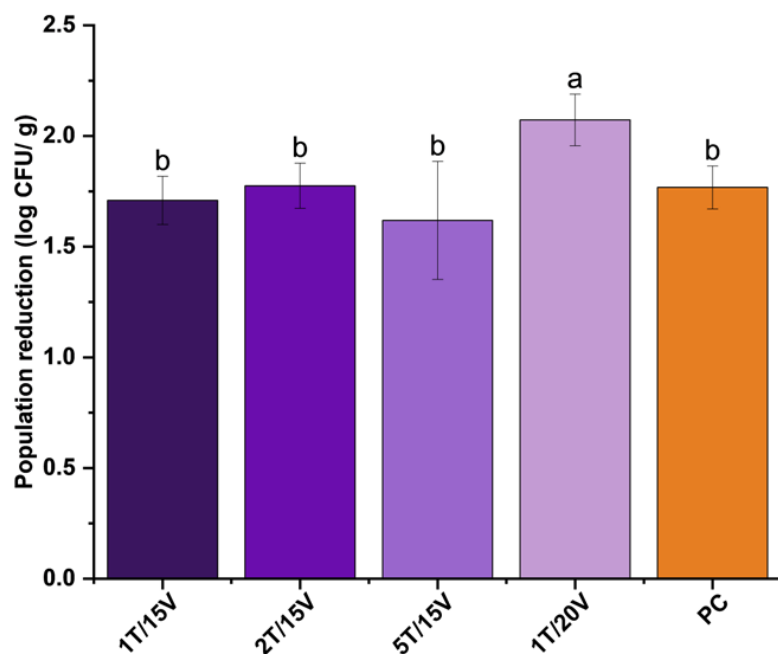


Figure 4.8: Microbial population reduction of HVACP-treated and untreated protein concentrates (Different lowercase letters show significant differences ($p < 0.05$) - one-way ANOVA ($n=2$)) (T-time; V-voltage; PC-untreated protein concentrate)

The initial microbial counts of the untreated protein concentrates were recorded to be very low, indicating near-sterile conditions of the samples. This could be attributed to several factors including the sterilizing effect that could have been implied by dehulling, which removes the outermost covering of the lentil seeds (Fandohan et al., 2006). In addition, the dry nature and the low water activity of the air classification process could also have stalled the growth of microorganisms in the protein concentrates.

One factor highlighted by the present study's results is the impact of voltage on microbial reduction. This is also evident Bahrami et al. (2016) in the findings of a study by Bahrami et al. (2016) on wheat flour, which failed to record a significant decrease in the population under lower-voltage treatment combinations. Extended treatment durations did not significantly enhance

microbial inactivation, as was also true in a study using turmeric powder. In this instance, microbial reduction was apparent only during the first 3 minutes and remained unchanged thereafter (Hemmati, Garavand, Goudarzi, et al., 2021). Further supporting the findings of the present study, Y. H. Kim et al. (2022) also reported that *E. coli* O157:H7 was not affected by extending the treatment time, indicating greater susceptibility to shorter treatment times, resulting in better population reduction. They also compared the inactivation effects of HVACP on *E. coli* O157:H7 and *Bacillus cereus*, concluding that HVACP is more effective against gram-negative bacteria. However, contrasting results were observed with green tea powder, where gram-positive bacteria showed greater susceptibility to treatment, and extended treatment led to greater reductions (Hemmati, Garavand, Khorshidian, et al., 2021). The authors suggested that the greater effect on gram-positive bacteria in the study is due to a change in the inactivation mechanism, related to the impact of reactive species on intracellular material.

The antimicrobial effectiveness of ACP is mainly attributed to the formation of reactive species during plasma discharge, such as ozone, superoxide anions, hydroxyl radicals, and nitrogen oxides. These reactive agents interact with microbial cell envelopes, disrupting membrane integrity and increasing permeability, which causes leakage of intracellular components. The resulting oxidative stress damages essential cellular constituents, including proteins, lipids, and nucleic acids, ultimately leading to microbial inactivation (Bahrami et al., 2016). Most studies indicate that the HVACP treatment is more effective against gram-negative bacteria, as they lack rigid peptidoglycan cell walls and are thus easily ruptured and affected by reactive species.

Overall, these findings highlight HVACP as a nonthermal technology capable of simultaneously enhancing protein functional properties while ensuring microbial safety, thereby supporting its broader application in plant-based protein ingredients.

4.5 Conclusion

This study demonstrates the potential of high-voltage atmospheric cold plasma (HVACP) as a multifunctional, nonthermal processing technology for plant protein ingredients. HVACP treatment induced controlled structural modifications in lentil protein concentrates, as evidenced by FTIR and SEM analyses, thereby improving key functional properties, including protein solubility, emulsifying activity, and foaming capacity across a range of pH conditions. Importantly, these functional enhancements were achieved without compromising the nutritional quality of the protein ingredients, as reflected by maintained or moderately improved in-vitro protein digestibility.

In addition to functional optimization, HVACP effectively reduced microbial populations by generating reactive oxygen and nitrogen species, highlighting its role in improving microbial safety and extending shelf life. The combined enhancement of protein functionality and microbial safety underscores HVACP's dual functionality and addresses critical challenges in plant-based protein systems, including limited digestibility and safety concerns. Overall, these findings position HVACP as a promising, sustainable processing strategy for developing safe, high-quality plant protein ingredients, while also underscoring the need for further systematic studies to optimize treatment conditions and elucidate underlying mechanisms.

Author contributions

Shimani Attygalle: Conceptualization, data curation, sample preparation, formal analysis, methodology, software, validation, visualization, writing-original draft, writing-review and editing

Mariela Rodriguez: Sample preparation, microbiological analysis, review and editing

Daniel Mayboca: Microbiological analysis, review and editing

Claudia Narvaez-Bravo: Microbiological analysis supervision, review and editing

Nandika Bandara: Conceptualization, funding acquisition, investigation, methodology, project administration, resources, software, supervision, editing

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Chapter 5: Conclusions and future research directions

The protein industry is actively seeking sustainable technologies to incorporate, given its rapid growth and rising consumer demand worldwide. Sustainability perspectives in the industry can be advanced through two main strategies: during protein extraction and modification. Accordingly, this study presents insightful findings on a sustainable extraction method, air classification, and a sustainable nonthermal protein modification method, cold plasma technology.

Summary of findings of Study 1: Optimizing Air Classification Conditions for Lentil Protein Concentrates for Functionality Improvement

- Dehulling and increasing the air classifier wheel speed significantly altered the crude protein, starch, and crude fat contents of concentrates
- Increased wheel speed reduced the particle size of fine fractions.
- Concentration of histidine, aspartic acid, proline, tyrosine, and tryptophan was significantly improved by increased wheel speed.
- Dehulling and increased wheel speed affected secondary structures, with α -helices decreasing and β turns increasing.
- Higher thermal stability was recorded for concentrates obtained from whole seeds.
- Scanning electron micrographs clearly demonstrated the differences in composition of fine and coarse fractions.
- Dehulling significantly affected the phytochemical composition, particularly phenolic and phytic acids.
- Techno-functional properties were significantly affected by both variables.
- Dehulling improved the in vitro protein digestibility (IVPD) of the concentrates.

Overall, dehulling and the air classifier wheel speed are critical factors influencing air classification efficiency and the properties of the resulting protein concentrates. These parameters should be carefully optimized based on the intended application.

Summary of findings of Study 2: Enhancing the Functionality of Lentil Protein Concentrates Using High Voltage Atmospheric Cold Plasma (HVACP)

- Increasing treatment time and voltage significantly increased carbonyl content, indicating enhanced protein oxidation, while concurrently reducing protein solubility at pH 7.
- Treatment combinations of 1 min/15 kV, 2 min/15 kV, 5 min/15 kV, and 1T/20 kV were optimal for treating lentil protein concentrates.
- Techno-functional properties were significantly affected, especially by the enhancement of the hydrophobic nature of protein molecules.
- The amino acid composition of Arginine, Glycine, Phenylalanine, and Tyrosine was affected by DBDCP treatment.
- Reduction in relative proportions of α -helices, and increases in β -sheets and aggregates were apparent upon treatment.
- DBDCP treatment reduced the thermal stability of concentrates.
- Morphological characteristics depicted a reduction in particle size and more surface etching as the treatment intensity increased.
- Phenolic acid content and in vitro protein digestibility significantly increased upon DBDCP treatment.
- In addition to improving functionality, DBDCP treatment exhibits dual functionality by reducing microbial load and enhancing the microbial safety of protein concentrates.

Overall, DBDCP under optimal conditions emerges as a promising approach to enhance the properties of protein concentrates, thereby helping overcome functionality-related challenges and promoting broader use of protein concentrates in the food industry.

Future research directions:

The current study comprehensively assessed the impact of dehulling as a pretreatment and air classification wheel speed as a process parameter on the properties of the resulting protein concentrate. Apart from dehulling, the effect of milling on the air classification efficiency can be studied in future studies, as it is known to have a considerable impact. Furthermore, air classification process parameters, such as the gas flow rate, can also be studied as variables. Another aspect to be considered is the effect of reclassification on the composition and functionality of the concentrates. Given the association of air classification with sustainability, developing Sankey diagrams and establishing energy and mass balances for the process will also be of great importance for fully understanding the potential of air classification.

The dielectric barrier discharge cold plasma system was also explored in detail during this study. Similar studies can be directed toward assessing the effects of different plasma generation systems or other process parameters of DBD cold plasma systems. These include gas composition, gas flow rate, distance between the sample and electrode, etc., and incorporating technologies to identify the reactive species formed and their shelf life in the product will also be valuable for providing mechanistic explanations of the changes observed in these studies.

Another avenue is to assess the impact on different legumes, beyond lentils, by considering varieties with varying chemical compositions. In addition, evaluating the performance of plasma-treated protein concentrates in complex food matrices would provide practical evidence of their

applicability in real food systems, including effects on product quality, stability, and sensory attributes. Finally, integrating air classification with complementary dry fractionation methods and conducting comprehensive sustainability and life cycle assessments would further strengthen the potential for industrial adoption of this combined, environmentally friendly processing approach.

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<https://doi.org/10.1016/j.fm.2014.02.007>

Supplementary Material

Supplementary Material 1: Amino acid composition and PDCAAS calculation of fine fractions compared with controls

AA	Requirement	Flour		NDH-30-F		NDH-40-F		DH-30-F		DH-40-F		Protein Isolate	
Composition	pattern ^a	AA	AAS ^c	AA	AAS ^c	AA	AAS ^c	AA	AAS ^c	AA	AAS ^c	AA	AAS ^c
(mg/g protein)	(mg/g protein)		(%)		(%)		(%)		(%)		(%)		(%)
Essential (EAA)													
Histidine (His)	19.00	20.46	1.08	20.94	1.10	23.89	1.26	20.99	1.10	22.57	1.19	23.44	1.23
Isoleucine (Ile)	28.00	39.27	1.40	44.15	1.58	44.67	1.60	45.31	1.62	45.42	1.62	51.36	1.83
Leucine (Leu)	66.00	67.58	1.02	77.22	1.17	78.24	1.19	78.24	1.19	79.45	1.20	90.80	1.38
Lysine (Lys)	58.00	63.83	1.10	72.14	1.24	72.66	1.25	72.38	1.25	74.00	1.28	77.29	1.33
Threonine (Thr)	34.00	34.77	1.02	36.29	1.07	36.44	1.07	36.70	1.08	36.70	1.08	40.33	1.19

Tryptophan (Trp)	11.00	7.75	0.70	8.46	0.77	8.80	0.80	8.68	0.79	8.61	0.78	9.04	0.82
Valine (Val)	35.00	43.91	1.25	48.86	1.40	49.81	1.42	49.69	1.42	50.73	1.45	56.29	1.61
Total	25.00	18.17	0.73	18.44	0.74	18.74	0.75	17.69	0.71	17.84	0.71	18.25	0.73
Sulphur amino acids (Met + Cys)													
Total	63.00	68.63	1.09	79.90	1.27	81.68	1.30	82.06	1.30	81.84	1.30	95.05	1.51
Aromatic amino acids (Phe + Tyr)													
LEAA ^b			TRP		MET+CYS		MET+CYS		MET+CYS		MET+CYS		MET+CYS
IVPD ^d			0.93		0.94		0.94		0.95		0.95		0.94
PDCAAS ^e			0.66		0.70		0.71		0.67		0.67		0.69
Non-essential (NEAA)													
Alanine (Ala)	-	66.95	-	78.05	-	78.84	-	81.09	-	81.29	-	88.53	-
Arginine (Arg)	-	109.4	-	122.4	-	124.0	-	122.9	-	124.6	-	135.3	-
		2		1		1		7		9		3	
Aspartic acid (Asp)	-	158.0	-	176.5	-	179.7	-	179.5	-	181.9	-	197.9	-
		0		0		3		0		3		2	

Glutamic acid (Glu)	-	36.23	-	38.85	-	39.52	-	39.64	-	39.58	-	42.36	-
Glycine (Gly)	-	42.34	-	44.54	-	45.04	-	44.91	-	45.18	-	46.93	-
Proline (Pro)	-	45.66	-	52.22	-	52.90	-	53.73	-	53.85	-	59.75	-
Serine (Ser)	-	66.95	-	78.05	-	78.84	-	81.09	-	81.29	-	88.53	-
Total EAA (mg/g protein)	-	364.4	-	406.4	-	414.9	-	411.8	-	417.2	-	461.9	-
Total NEAA (mg/g protein)	-	499.9	-	555.3	-	562.6	-	564.1	-	569.8	-	617.6	-
Total AA (mg/g sample)	-	217.1	-	493.7	-	521.0	-	499.5	-	535.9	-	901.3	-

(NDH-Whole seeds; DH-Dehulled seeds; 30- 30 Hz; 40- 40Hz; F- Fine fraction) ^aRecommended essential amino acid pattern for a pre-school child (2-5years, mg/g protein) (FAO/WHO, 1991) ^bLimiting essential amino acid; ^cAmino acid score = amino acid content of test protein/ ref AA pattern; ^dIVPD = In vitro protein digestibility; ^ePDCAAS = AAS*IVPD

Supplementary Material 2: Amino acid composition and PDCAAS calculation of HVACP treated and untreated samples

AA Compositio n (mg/g protein)	Requiremen t pattern ^a (mg/g protein)	1T/15V		2T/15V		1T/20V		PC	
		AA	AAS	AA	AAS	AA	AAS	AA	AAS
Essential (EAA)									
Histidine (His)	19	40.07 ± 1.16 ^a	2.11	38.78 ± 0.26 ^a	2.04	40.29 ± 1.39 ^a	2.12	38.51 ± 0.56 ^a	2.03
Isoleucine (Ile)	28	46.26 ± 0.08 ^a	1.65	45.24 ± 0.47 ^a	1.62	47.16 ± 1.26 ^a	1.68	45.87 ± 0.38 ^a	1.64
Leucine (Leu)	66	79.62 ± 0.34 ^a	1.21	77.87 ± 0.58 ^a	1.18	79.05 ± 0.00 ^a	1.20	77.99 ± 1.27 ^a	1.18
Lysine (Lys)	58	74.73 ± 0.35 ^a	1.29	73.42 ± 0.16 ^a	1.27	76.05 ± 1.02 ^a	1.31	74.01 ± 0.85 ^a	1.28
Threonine (Thr)	34	39.56 ± 0.42 ^a	1.16	38.75 ± 0.16 ^a	1.14	40.00 ± 0.71 ^a	1.18	39.02 ± 0.21 ^a	1.15
Tryptophan (Trp)	11	8.38 ± 0.15 ^a	0.76	8.05 ± 0.05 ^a	0.73	8.43 ± 0.16 ^a	0.77	8.26 ± 0.21 ^a	0.75
Valine (Val)	35	51.53 ± 0.33 ^a	1.47	50.51 ± 0.53 ^a	1.44	52.09 ± 1.13 ^a	1.49	50.59 ± 0.33 ^a	1.45
Total Sulphur amino acids	25	19.40 ± 0.68 ^a	0.78	19.52 ± 0.24 ^a	0.78	17.52 ± 0.13 ^b	0.70	18.70 ± 0.14 ^{a^b}	0.75

(Met + Cys)									
Total Aromatic amino acids									
(Phe + Tyr)	63	89.89 ± 0.05 ^a	1.43	87.85 ± 0.41b ^c	1.39	89.17 ± 0.00a ^b	1.42	86.83 ± 0.63 ^c	1.38
LEAA ^b		TRP		TRP		MET + CYS		MET + CYS	
IVPD ^d		95.33		95.22		95.44		94.54	
PDCAAS ^e		72.63		69.68		66.89		70.72	
Non-essential (NEAA)									
Alanine									
(Ala)	-	43.66 ± 0.04a ^b	-	42.92 ± 0.16 ^b	-	44.10 ± 0.39 ^a	-	43.38 ± 0.06a ^b	-
Arginine									
(Arg)	-	84.45 ± 0.00 ^a	-	83.50 ± 0.18a ^b	-	83.63 ± 1.85a ^b	-	80.66 ± 0.00 ^b	-
Aspartic acid									
(Asp)	-	116.67 ± 0.90a ^b	-	115.81 ± 0.64 ^b	-	119.71 ± 0.59 ^a	-	115.93 ± 1.16 ^b	-
Glutamic acid (Glu)									
(Glu)	-	169.88 ± 0.87a ^b	-	167.46 ± 0.93 ^b	-	173.04 ± 1.14 ^a	-	168.57 ± 1.03 ^b	-
Glycine									
(Gly)	-	40.92 ± 0.33 ^a	-	38.46 ± 0.02 ^b	-	40.83 ± 0.90 ^a	-	40.20 ± 0.44 ^b	-
Proline (Pro)									
(Pro)	-	46.61 ± 0.44 ^a	-	45.47 ± 0.28 ^a	-	46.92 ± 0.87 ^a	-	45.67 ± 0.55 ^a	-
Serine (Ser)									
(Ser)	-	54.10 ± 0.47 ^a	-	54.41 ± 0.51 ^a	-	56.54 ± 1.29 ^a	-	54.31 ± 0.49 ^a	-

Total EAA (mg/g protein)	-	449.45 ± 3.57 ^a	-	439.97 ± 0.63 ^a	-	449.74 ± 5.22 ^a	-	439.79 ± 1.79 ^a	-
Total NEAA (mg/g protein)	-	556.29 ± 0.56 ^a ^b	-	548.03 ± 1.75 ^b	-	564.77 ± 7.02 ^a	-	548.73 ± 1.75 ^b	-
Total AA (mg/g sample)	-	520.49 ± 1.56 ^a	-	514.07 ± 0.58 ^a	-	518.34 ± 6.25 ^a	-	510.72 ± 0.02 ^a	-

(Different lowercase letters compare between samples for each row, show significant differences (p<0.05) - one-way ANOVA (n=2))
(T-time; V-voltage; PC-untreated protein concentrate) ^aRecommended essential amino acid pattern for a preschool child (2-5years, mg/g protein) (FAO/WHO, 1991) ^bLimiting essential amino acid; ^cAmino acid score = amino acid content of test protein/ ref AA pattern;
^dIVPD = In vitro protein digestibility; ^ePDCAAS = AAS*IVPD

