# Pea Protein Globulins: Does their Relative Ratio Matter?

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Holly Francine Husband

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# Dedication

To Kylo's care team – thanks for being there for both of us.

## Abstract

The relatively low abundance of 11S legumin in pea protein, coupled with the wide diversity in 7S vicilin to 11S legumin ratio among pea protein ingredients, are assumed contributors to pea protein's inferior and inconsistent functionality and nutritional quality relative to soy protein. To improve the performance of pea protein ingredients in food and beverage applications, optimum protein profile must be identified. Therefore, this work followed a holistic approach to determine the impact of 7S/11S ratio on pea protein structure, functionality, and nutritional quality. Vicilin- and legumin-rich fractions were isolated and combined in different proportions to produce pea protein samples of varying 7S/11S ratios. For the first time, pea protein isolate was also enriched with 11S legumin to evaluate the impact of 11S abundance on functionality within an unfractionated protein matrix. Results revealed the isolated 7S vicilin had 6 fold higher gel strength and 5 fold higher emulsification capacity, but significantly lower nutritional quality, than the isolated 11S legumin. Despite having significantly higher sulfur-containing amino acids, high protein polymerization in the isolated 11S legumin contributed to the relatively low functionality. Further, fractionation induced unique changes to amino acid composition, resulting in significantly lower amino acid scores for isolated 7S vicilin and 11S legumin relative to pea protein isolate. Accordingly, 11S legumin enrichment of pea protein isolate did not improve functionality or nutritional quality. Nevertheless, this work contributed foundational knowledge that will provide direction for future studies aiming at devising strategies to improve the quality and consistency of pea protein ingredients.

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## 1.1 Introduction

In the United States, grocery sales of plant-based foods grew 44% in the last three years and have reached a market value of $8 billion (SPINS/GFI, 2023). Among plant-based products, soy protein is the principal protein ingredient across categories (McClements and Grossmann, 2022). The soy protein dominance in the plant protein market is attributed to many decades of research that led to a comprehensive understanding of its excellent functionality and nutritional quality (Kimura et al., 2008; McClements and Grossmann, 2022). However, pea protein is attracting interest as a soy protein replacement due to its current non-allergenic, non-genetically modified status. In fact, pea protein is one of the fastest growing plant proteins in global alternative product launches, with its rampant growth attributed to the agronomic benefits of growing pea, low production cost, and acceptable nutritional quality (Barac et al., 2010; Grand View Research, 2023a).

As legumes, pea and soy have considerable homology in their protein components (Danielsson, 1949; Schroeder, 1982). The major protein components in pea, 7S vicilin and 11S legumin, have similar molecular weight, amino acid composition, and subunit structures to their counterparts in soy, 7S ß-conglycinin and 11S glycinin (Derbyshire, Wright, and Boulter 1976). The 7S and 11S proteins are largely responsible for protein functionality and nutritional quality in foods (Gueguen and Barbot, 1988; Tulbek, Lam, Wang, Asavajaru, and Lam, 2017).

Despite their similarities in protein profile, pea protein has inferior functionality in food and beverage applications in comparison to soy protein (Zhao, Shen, Wu, Zhang,

and Xu, 2020). This inferiority may be in part explained by the differences in the 7S/11S ratio between pea and soy. In soy protein, the ratio of 7S/11S may range from 0.47-0.79 across cultivars (Murphy and Resurreccion, 1984; Tzitzikas, Vincken, De Groot, Gruppen, and Visser, 2006). This regularity in protein profile among soy cultivars, coupled with the larger abundance of the highly functional 11S glycinin over 7S ß-conglycinin, have led to consistent functionality and nutritional quality of soy protein ingredients (Rutherfurd, Fanning, Miller, and Moughan, 2014; Tzitzikas et al., 2006). Meanwhile, a much wider diversity in the 7S/11S ratio in pea has been reported, ranging from 0.2-8.0 depending on factors such as agronomic practices, environmental conditions, and most importantly, genetic origin (Casey, Sharman, Wright, Bacon, and Guldager, 1982; Mertens, Dehon, Bourgeois, Verhaeghe-Cartrysse, and Blecker; 2012). These factors also affect protein subunit composition and conformation, which contribute to pea protein heterogeneity beyond the 7S/11S ratio (Derbyshire et al., 1976). The culmination of these variances has significant implications on pea protein functionality and nutritional quality (Casey et al., 1982; Gueguen and Barbot, 1988). In fact, Barac et al. (2010) and O'Kane, Vereijken, Gruppen, and Van Boekel (2005) reported that pea proteins from different genotypes had significantly different emulsifying and gelling properties, respectively. In addition to functional heterogeneity, the protein digestibility corrected amino acid score (PDCAAS) of pea protein fluctuated between 0.54-0.89 among different cultivars (Mertens et al., 2012; Nosworthy and House, 2017; Rutherfurd et al., 2014).

These observations inspired researchers to investigate the relationship between pea protein profile and functional behavior. Dagorn-Scaviner, Gueguen, and Lefebvre

(1986, 1987) mixed pea 7S vicilin and 11S legumin in four different 7S/11S ratios (0.33, 0.5, 1.0, and 3.0), and determined that vicilin had greater surface activity and emulsifying properties than legumin. In contrast, Koyoro and Powers (1987) found that purified legumin had higher emulsification capacity than purified vicilin and a 7S/11S mixture (unspecified ratio). Further, Bora, Brekke, and Powers (1994), who prepared one 7S/11S ratio (1.8) from purified fractions, reported that vicilin-rich samples had good gelling properties, while legumin formed weak gel structures. On the other hand, O'Kane, Happe, Vereijken, Gruppen, and Van Boekel (2004a) prepared three 7S/11S ratios (0.22, 0.57, and 1.2) and found that legumin formed thermally induced gels, while certain vicilin subunits inhibited gelation.

Because of these contradictory findings, the impact of pea protein profile on functional behavior remains unclear. Therefore, characterization of pea 7S vicilin, 11S legumin, and their ratios, beyond what has been reported thus far, is needed. Furthermore, the current knowledge of 11S legumin functionality has largely been acquired based on its isolated and purified form, where no studies have evaluated the functional effects of enriching a pea protein isolate with 11S legumin. Such investigation is necessary to understand how the inherent components and environmental factors introduced by the original pea protein matrix affect the protein-protein interactions and resultant functional behavior of 11S legumin in varying abundance. Furthermore, the intrinsic variance in amino acid composition of pea 7S vicilin and 11S legumin necessitates more evidence relating pea protein profile with nutritional quality. This knowledge is critical to address the lower and inconsistent nutritional quality of pea protein compared to soy protein.

The aforementioned variability in pea protein and its functionality, which goes beyond differences in protein extraction and processing conditions, continues to present formulation and consistency challenges to the food industry. Therefore, to successfully incorporate pea protein into different food and beverage applications and limit inconsistencies, differences in the protein profile and the consequent impact on functionality need a holistic investigation. Outcomes of a thorough investigation may also contribute to targeted breeding strategies for the continual development of pea as a source of functional and nutritious protein.

### 1.2 Hypothesis and objectives

The protein profile of an ingredient is an indicator of its performance in food and beverage applications. For example, the high functionality and nutritional quality of soy protein is attributed to the high abundance of 11S glycinin relative to 7S ß-conglycinin. In contrast, pea protein has an inherently low 11S legumin to 7S vicilin ratio. It is hypothesized that the difference in 7S/11S ratio between soy and pea protein is largely responsible for the latter's relatively inferior functionality and nutritional quality. Additionally, as 11S glycinin's unique molecular and structural characteristics contribute significantly to soy protein's high functionality, it is hypothesized that higher 11S legumin abundance in pea protein will enhance pea protein functionality. Lastly, as 11S legumin compared to 7S vicilin has a higher abundance of cysteine and methionine, the limiting amino acids in pea protein, than 7S vicilin, it is hypothesized that a higher proportion of 11S legumin in pea protein will enhance its nutritional quality. Therefore, the main objective of this work is to determine the impact of varying the proportion of 7S

vicilin and 11S legumin on the functional behavior and nutritional quality of pea protein. Specific goals of this work include:

1. Produce enriched fractions of 7S vicilin and 11S legumin from pea flour.
2. Determine the impact of each fraction and selected 7S/11S ratios, in the isolated form and in the native pea protein matrix, on the structural, functional, and nutritional quality of pea protein.

### 1.3 Plant protein ingredients demand and market

Demand for protein on a global scale has rapidly increased in recent years and is expected to continue climbing. In 2022, the global protein ingredient market was valued at $77.7 billion and was expected to grow at a compound annual growth rate (CAGR) of 5.8% from 2023 to 2030 (Grand View Research, 2023b). This momentous demand reflects the importance of protein to consumers. Protein is a fundamental component of human nutrition for its role in various physiological processes supporting human growth and development. For example, protein serves as a cellular structural component, is a key participant in energy metabolism, and forms the major constituents of muscle (Institute of Medicine, 1999). Adequate protein intake is necessary to prevent protein-energy malnutrition, which has a serious disease burden especially among children, the elderly, and in populations of lower socioeconomic status (Zhang et al., 2022). On the other hand, in Westernized countries where metabolic disease is more prevalent, protein is important to support weight loss efforts, glycemic regulation, and satiety (Pasiakos, 2015). Protein is valued even among healthy individuals for its role in promoting healthy aging, namely improved bone health, muscle regeneration and maintenance, and enhanced immune

function (Ismail, Senaratne-Lenagala, Stube, and Brackenridge, 2020; Sathe, Zaffran, Gupta, and Li, 2018).

Among the different sources of dietary protein, animal-based proteins accounted for 79% of the share in global protein revenue in 2022 (Grand View Research, 2023b). Consumers have historically preferred animal-based proteins for their taste and nutrition (Ismail et al., 2020). There are also social and emotional components to consumption of animal protein. Surveys revealed that meat elicits an emotional response from consumers, with some consumer groups believing that meat is indicative of social status and/or that it is a necessary component of every meal (Bryant, 2022; Chychula, 2022). However, despite the pronounced importance of animal-based protein to consumers, there is concern over the viability of animal agriculture in sustaining the human food supply. These apprehensions have become more widespread and urgent in consideration of the projected 10 billion global population by 2050 (McClements and Grossmann, 2022). Animal agriculture has been linked to significant greenhouse gas emissions, deforestation, land erosion, pollution, biodiversity loss, and zoonotic disease transmission (McClements and Grossmann, 2022; Nielsen, 2022). On the other hand, researchers have reported plant-based products generally require less agricultural land and water than livestock and cause considerably less pollution and biodiversity loss (McClements and Grossmann, 2022; Nielsen, 2022; Willett, Rockstrom, and Loken, 2019). Accordingly, diet trends including veganism, vegetarianism, or flexitarianism have become more popular among consumers wishing to reduce their meat intake and help alleviate the environmental and ethical cost of animal agriculture (Nielsen, 2022).

Other than environmental and animal welfare concerns, health is one of the most important drivers for consumer purchase of plant-based products (Corrin and Papadopoulos, 2017; Fox and Ward, 2008; Nielsen, 2022). There are 200 million shoppers in the United States who adhere to a diet or health-related program, and many believe consumption of plant-based proteins will enhance their health status (Bryant, 2022*)*. Such consumer beliefs have been supported by certain studies that found a plant-based diet could reduce the incidence of health disorders such as obesity, digestive problems, and diabetes (Campbell, 2006; Greger, 2015; Willett et al., 2019). In addition to health, factors such as taste, cost, variety, and convenience drive consumer purchase decisions for plant-based products (International Food Information Council, 2021).

In response to shifting consumer demand for plant-based products, the food industry has expanded its plant-based offerings across categories, leading to significant growth in the plant-based market over the past few years. Accordingly, the plant-based market has reached a market value of $8 billion in the United States and has a CAGR of 9% (Grand View Research, 2023b; SPINS/GFI, 2023). Moreover, grocery sales of plant-based foods that directly replace animal products have grown 44% in the past three years, with plant-based milk, other plant-based dairy, and plant-based meat as the top grossing categories (SPINS/GFI, 2023). Proteins are the most important functional ingredient in products across these categories due to their ability to provide unique structuring, texturizing, emulsifying, foaming, fluid holding, and nutritional attributes (Ismail et al., 2020; McClements and Grossmann, 2022; Sim, Srv, Chiang, and Hensry, 2021). Therefore, it is imperative for the food industry to understand the functionality and

nutritional quality of different plant proteins in food and beverages to satisfy consumer expectations for plant-based products.

Among plant proteins, soy protein has historically been a leading contributor to the formulation of many plant-based products across categories (Kimura et al., 2008; McClements and Grossmann, 2022). The popularity of soy protein can be attributed to a variety of reasons, including its positive agronomic traits, low cost, and wide availability (Barac et al., 2010). Further, decades of research have led to a comprehensive understanding of soy protein functionality and nutritional quality, enabling its expanded use in various food and beverage products (Rickert, Johnson, and Murphy, 2004). The protein functional behavior and nutritional quality are dependent on the inherent characteristics. Therefore, it is important to understand how the inherent profile of soy protein contributes to its expanded application in the food and beverage industry.

### 1.4 Soy protein profile

Soy protein is mostly composed of water-soluble 2S albumins and salt-soluble 7S and 11S globulins (Murphy and Resurreccion, 1984). Albumins encompass between 10-20% of the total protein and include enzymes, protease inhibitors, amylase inhibitors, lectins, and other metabolic proteins, which are non-functional in food applications (Boye, Zare, and Pletch, 2010; Lam, Warkentin, Tyler, and Nickerson, 2017). Meanwhile, 7S β-conglycinin and 11S glycinin comprise between 28-41% and 38-51% of the total protein, respectively (Murphy and Resurreccion, 1984). Each of these globulins individually impact soy protein functionality and nutritional quality due to their unique amino acid profile, size, and structure (Gueguen and Barbot, 1987; Khatib,

Herald, Aramouni, MacRitchie, and Schapaugh, 2022; Nagano, Fukuda, and Akasaka, 1996; Riblett, Herald, Schmidt, and Tilley, 2001; Rickert et al., 2004; Tulbek et al., 2017). Therefore, the unique characteristics of each globulin will be discussed.

### 1.4.1 11S Glycinin

Glycinin is a hexameric protein with a molecular weight of 300-380 kDa and a sedimentation coefficient of 11S (Tang, 2017). Its quaternary structure is formed through the association of 2 trimers by electrostatic and hydrophobic interactions. Each trimer is made up of 3 monomers associated via strong hydrophobic interactions (Adachi et al., 2003).

Five major monomers have been identified in glycinin according to their primary structure: A1aB1b, A1bB2, A2B1a, A3B4, and A5A4B3 (Fukushima, 2011). These monomers can be classified into either Group 1 or Group 2 according to the homology in their sequences (Kimura et al., 2008). Group 1 monomers, A1aB1b, A1bB2 and A2B1a, are uniform in size (~58 kDa), contain a higher amount of methionine, and display 90% homology across group members (Nielsen, 1985). Meanwhile, group 2 monomers (A3B4 and A5A4B3) are larger (~62-69 kDa) and contain less methionine than the group 1 monomers (Nielsen, 1985). The number of amino acid residues, especially charged residues, are different among the five monomers, which impact the surface properties and functional behavior of both the individual monomers and the assembled glycinin oligomer (Nielsen, 1985; Prak, Nakatani, Katsube-Tanaka, Adachi, Maruyama, and Utsumi, 2005; Tezuka, Taira, Igarashi, Yagasaki, and Ono, 2000). Researchers have examined the structure-function relationship of each glycinin monomer (Maruyama et al.,

2002, 2004; Rickert, Johnson, and Murphy, 2004) but only that of the assembled glycinin molecule will be discussed further.

Glycinin, as compared with other soy protein globulins, is unique in that its monomers contain an acidic (~40 kDa) and basic (~20 kDa) polypeptide linked by a single disulfide bond (O'Kane et al., 2004c). In addition to these 6 inter-molecular disulfide linkages, glycinin contains 15 intra-molecular disulfide bonds (Tang, 2011). The presence of disulfide bonds contributes to glycinin's rigid and compact quaternary structure, reducing its molecular flexibility (Rickert et al., 2004). Glycinin's low molecular flexibility contributes to its relatively low foaming and emulsifying properties when in native form (Wagner and Gueguen, 1995).

Glycinin's thermal stability, on the other hand, impacts gel formation. A relatively high temperature (~90ºC) is needed for denaturation of glycinin, which subsequently induces hydrophobic protein association and gel network formation (Tang, 2011). However, glycinin's high abundance of disulfide bonds and cysteine residues allow the formation of a fine-stranded, uniform network, which enhances structural stability of the gel (O'Kane et al., 2004c; Rickert et al, 2004). The notable gelling behavior of glycinin is often a highlighted component of soy protein functionality, as it improves the firmness, pliability, and texture of foods such as meat and cheese analogues (Kinsella, 1979; McClements and Grossmann, 2022).

### 1.4.2 7S β-conglycinin

β-conglycinin is a trimer with a molecular weight of 150-200 kDa and a sedimentation coefficient of 7S (Fukushima, 2011). β-conglycinin's quaternary structure

is formed mainly through hydrophobic association of three subunits, α (68 kDa), α' (72 kDa), and β (52 kDa) (Thanh and Shibasaki, 1977). Molecular heterogeneity within β-conglycinin trimers has been reported, as seven molecular species including α′β2, αβ2, αα′β, α2β, α2α′, α3 and β3 have been identified (Fukushima, 2011). Despite this heterogeneity, the core regions of all three subunits exhibit high homologies with each other, are glycosylated via asparagine residues, and are devoid of disulfide bonds (Hirano, Kagawa, Kamata, and Yamauchi, 1987; Murphy, 2008; Tandang-Silvas et al., 2010). The α and α' subunits are unique from the β subunit as they contain extension regions that are rich in acidic amino acid residues (Kimura et al., 2008).

The high molecular flexibility of β-conglycinin, as compared to glycinin, may be attributed to its relatively higher surface hydrophobicity and lack of disulfide linkages (Hayakawa and Nakai, 1985; Murphy, 2008). The molecular flexibility and less compact structure of β-conglycinin compared to glycinin contribute to lower denaturation temperature ($T_d$ ~80ºC) and better emulsifying and foaming properties (Rickert et al., 2004). β-conglycinin's carbohydrate moieties and extension regions further enhances its surface activity and its solubility at neutral pH (Kimura et al., 2008). On the other hand, the lack of disulfide linkages and cysteine residues in β-conglycinin reduces its ability to form a strong gel network (Wu, Hua, Chen, Kong, and Zhang, 2017). Nevertheless, the unique functional behavior of β-conglycinin is valuable in applications such as salad dressings and whipped toppings (McClements & Grossmann, 2022).

### 1.4.3 7S/11S ratio in soy protein

Though β-conglycinin and glycinin uniquely contribute to the functionality and nutritional quality of soy protein, their relative ratio in a protein ingredient is an important indicator of the overall functionality and nutritional quality. The ratio of 7S/11S in soy protein typically falls within the narrow range of 0.47-0.79 across cultivars (Murphy and Resurreccion, 1984; Tzitzikas et al., 2006). The regularity in protein profile among soy cultivars, coupled with the larger abundance of the highly functional 11S glycinin over 7S β-conglycinin, have led to consistent functionality and nutritional quality of soy protein ingredients (Rutherfurd et al., 2014; Tzitzikas et al., 2006). Further, years of breeding efforts to eliminate molecular variability within soy protein have contributed to consistent functional properties, such as solubility, gelling performance, and emulsification capabilities, as well as nutritional quality (Mertens et al., 2012; Murphy and Resurreccion, 1984).

## 1.5 Limitations of soy protein

Although soy protein delivers consistent functionality and nutritional quality, there are perceived drawbacks to its use in plant-based foods. For example, soy protein is among the top nine allergens in the United States (FDA, 2022). Additionally, over 90% of soybeans in the United States are produced using genetically engineered varieties (USDA, 2022). These factors have caused an unprecedented consumer apprehension toward soy and have prompted the food industry to develop plant-based products without their historic reliance on soy protein ingredients (Ismail et al., 2020). Therefore, it is

necessary to identify consumer acceptable, industry feasible alternatives to soy protein that will deliver high functionality and nutritional quality in plant-based products.

### 1.6 Emergence of pea protein as a potential soy protein replacement

Peas are cool-season pulse crops cultivated predominantly for protein and animal feed purposes (Pavek, 2012; Tulbek et al., 2017). Peas are inherently nutrient dense, containing rich amounts of protein, starch, and dietary fiber, while having low fat content (Dahl, Foster, and Tyler, 2012; Tzitzikas et al., 2006). Peas are also a good source of minerals and are particularly high in potassium, magnesium, and calcium (Reichert and MacKenzie, 1982). Antinutritional components inherent to pea, such as trypsin inhibitors, phytic acid, and oligosaccharides, may limit the bioavailability of nutrients, but processing may minimize or eliminate their effects (Wang and Daun, 2004).

Peas can be milled and fractionated to provide an array of ingredients including flours, protein concentrates, protein isolates, starches, and fibers (Tulbek et al., 2017). Typically, dry-milling or wet-milling techniques are followed to produce pea protein ingredients with protein content between 48% and 90% (Tulbek et al., 2017). While different classes of field pea are available, yellow field pea (*Pisum sativum L.*) is commonly used to produce these pea protein ingredients (Lu, He, Zhang, & Bing, 2020; Tulbek et al., 2017).

Pea protein has attracted specific interest as a soy protein replacement due to its current non-allergenic, non-genetically modified status. In fact, pea protein is one of the fastest growing plant proteins in global alternative product launches, with its market value forecasted to reach $4.7 billion by 2030 (Grand View Research, 2023a). This

rampant growth is attributed to the agronomic benefits of growing pea, low production cost, and acceptable nutritional quality (Barac et al., 2010).

Though pea protein ingredients are currently utilized in beverages, bakery products, pasta, extruded snacks, and meat analogs, its lower solubility, emulsification, and gelling properties relative to soy protein present challenges in these applications (Hansen, Bu, and Ismail, 2022; Tulbek et al., 2017; Zhao et al., 2020). Further, substantial variation in the functional properties and nutritional quality of pea protein ingredients has been identified, making the manufacture of products with consistent characteristics challenging for food processors (Choi, Taghvaei, Smith, and Ganjyal, 2022; Taghvaei, Sadeghi, and Smith, 2022). As the functional behavior and nutritional quality of protein ingredients are related to protein profile, it is important to examine the inherent composition of pea protein to identify opportunities for its expanded application in the food and beverage industry.

### 1.7 Pea protein profile

Pea protein is primarily composed of albumins and globulins (15-25% and 50-60% of total protein, respectively) and contains minor amounts (< 5% of total protein) of prolamins and glutelins (Boye et al., 2010; Gueguen and Barbot, 1988; Saharan and Khetarpaul, 1994). Albumins are metabolic proteins and are non-functional in food applications, as discussed. The globulin proteins in pea include 11S legumin, 7S vicilin, and 8S convicilin, which typically account for 6-25%, 26-52%, and 4-8% of the total protein, respectively (Lam, Can Karaca, Tyler, and Nickerson, 2018; Tzitzikas et al.,

2006). These globulins are pertinent to pea protein functionality and nutritional quality, and the unique characteristics of each will be discussed.

### 1.7.1 11S Legumin

Legumin is a hexameric protein with a molecular weight of 300-400 kDa and a sedimentation coefficient of 11S (Lam et al., 2018). Legumin's quaternary structure is formed through the association of 2 trimers by electrostatic and hydrophobic interactions (Tzitzikas et al., 2006). Each trimer contains 3 monomers associated via strong hydrophobic interactions (Sun and Arntfield, 2012; Tzitzikas et al., 2006), and each monomer contains an acidic (~40 kDa) and basic (~20 kDa) subunit linked by an interchain disulfide linkage (Barac et al., 2010). Monomers can be further classified into the legumin families LegA, LegJ, or LegS based on the homology of the peptide sequence. LegA and LegJ families encompass monomers with molecular weights between 60-65 kDa, while those of LegS have a molecular weight of ~80 kDa (Barac et al., 2010; Tzitzikas et al., 2006).

Unique amino acid compositions have been identified in the acidic and basic subunits of legumin. A relatively high abundance of tyrosine, glutamic acid, and leucine residues exists in the acidic subunit (Sikorski, 2001). The acidic subunit also contains approximately 2 cysteine residues per ~60 kDa monomer (Casey and Short, 1981). On the other hand, the basic subunit has a relatively greater abundance of alanine, valine, leucine, and glycine residues, and it contains approximately 3 methionine residues per ~60 kDa monomer (Casey and Short, 1981; Sikorski, 2001). The distinct amino acid composition of the acidic and basic subunit contributes to their relatively high

hydrophilicity and hydrophobicity, respectively (Gueguen and Barbot, 1988). Consequently, the acidic polypeptide is predominantly found on the surface of the legumin molecule, while the basic polypeptide is often buried within the inner moiety of the legumin molecule (Gueguen and Barbot, 1988).

The inherent size, structure, and amino acid composition of legumin have several implications on its nutritional quality and functionality. Legumin has a higher abundance of sulfur-containing amino acids relative to the other pea protein globulins, which enhances its nutritional quality (Casey and Short, 1981; Millerd, Thomson, and Schroeder, 1978). The cysteine residues in legumin indicate a propensity for the formation of disulfide bridges, which is a prerequisite for thermal gelation (Rickert et al., 2004). However, legumin's intermolecular disulfide bridges, coupled with its complex quaternary structure, result in a rigid conformation which reduces its susceptibility to denaturation (Mession, Chihi, Sok, and Saurel, 2015). Accordingly, legumin denatures at a higher temperature (~90°C) compared to other globulins (Kornet et al., 2021). Resistance to denaturation may affect functional properties such as thermal gelation, emulsification, and foaming, as previously described (Mession et al., 2015). Further, legumin's relatively high abundance of surface hydrophobic groups affects hydrophobic, electrostatic, and steric parameters of the protein, which collectively influence its functional behavior (Barac et al., 2010; Mwasaru, Muhammad, Bakar, and Che Man, 1999; Nakai, 1983). For example, high surface hydrophobicity is often correlated with emulsifying capacity and with thermal functional properties (heat coagulation, gelation, and thickening), but may reduce protein solubility (Nakai, 1983, Shimada and Matsushita, 1980). Modification of legumin or its environment to alter the charge

frequency, hydrophobicity, and structure of the protein can change its functionality (Nakai, 1983).

### 1.7.2 7S Vicilin

Vicilin is a trimer with a molecular weight of 150-180 kDa and a sedimentation coefficient of 7S (Barac et al., 2010; Sikorski, 2001). Vicilin's quaternary structure is formed through the association of three monomers (~47-50 kDa) by hydrophobic interactions (Lam et al., 2018). Vicilin fragments (α, β, γ) are generated from post-translational proteolytic processing of the intact monomer (Gatehouse, Lycett, Croy, & Boulter, 1982; Pedrosa and Ferreira, 1994). Depending on the site of post-translational cleavage, polypeptides with molecular weights of ~50 kDa, 30-35 kDa and ≤19 kDa are produced, but they remain associated via noncovalent interactions (Casey and Domoney, 1999; Croy, Gatehouse, Tyler, and Boulter 1980; Davey and Dudman, 1979; Gatehouse, Croy, Morton, Tyler, and Boulter, 1981). Further, Gatehouse et al. (1981, 1982) found N-glycosylation close to the C terminus of the γ fragment (~12-16 kDa). This finding agreed with Boulter (1979), who identified pea vicilin as a glycoprotein, containing 2-5% carbohydrate.

Vicilin's inherent characteristics impact its nutritional quality and functional properties. Although vicilin contains high levels of arginine, lysine, aspartic acid, and glutamic acid, it is deficient in tryptophan, methionine, and cysteine, which limits its nutritional quality (Barac et al., 2010; Jackson, Boulter, and Thurman, 1969; Lam et al., 2018; Sikorski, 2001). Vicilin's lower molecular weight, glycosylated subunits, and conformational flexibility typically contribute to high solubility, foaming, and

emulsifying properties (Kimura, Fukuda, Zhang, Motoyama, Maruyama, and Utsumi, 2008; Maruyama et al., 2002; Pedrosa, Trisciuzzi, and Ferreira, 1997). However, vicilin cannot form inter- or intra-chain disulfide linkages due to its lack of cysteine residues, which results in lower denaturation temperature (~80°C) and gelation potential compared to legumin (Rickert et al., 2004).

### 1.7.3 8S Convicilin

Convicilin has a molecular weight between 210-290 kDa and contains either three or four subunits of 71 kDa (Croy et al., 1980; Gatehouse et al., 1981; Tzitzikas et al., 2006). Although convicilin and vicilin show considerable homology in their amino acid sequence, convicilin is differentiated in a few ways. First, convicilin contains unique encoding genes that prevent post-translational modification (Bown, Ellis, and Gatehouse, 1988; Domoney and Casey, 1990; Newbigin et al., 1990; O'Kane, Happe, Vereijken, Gruppen, and Van Boekel, 2004b). Further, each subunit of convicilin contains one cysteine residue and one methionine residue (Boye et al., 2010; Croy et al., 1980; O'Kane et al., 2004b). Lastly, convicilin possesses a N-terminal extension that is highly charged with acidic residues and lacks hydrophobic residues (O'Kane et al., 2004b; Tzitzikas et al., 2006).

There are limited studies on the functionality and nutritional quality of convicilin. Some researchers determined that convicilin is relatively insoluble, despite its highly charged N-terminal extension (Casey and Sanger, 1980; O'Kane et al., 2004b). Convicilin's charged terminus was also thought to inhibit protein gelation (O'Kane et al., 2004a). However, in consideration of the inherently low abundance of convicilin relative

to other globulins in pea protein, its individual contribution to overall functionality and nutritional quality could be insignificant.

### 1.7.4 Structural-functional comparison of 7S and 11S globulins between pea and soy

As legumes, pea and soy have considerable homology in their protein components (Danielsson, 1949; Schroeder, 1982). The major protein components in pea, 7S vicilin and 11S legumin, have similar molecular weight, amino acid composition, and subunit structures to their counterparts in soy, 7S β-conglycinin and 11S glycinin (Derbyshire et al., 1976). Despite these similarities, distinct physicochemical characteristics, and resultant functional behavior, have been reported for the globulins across the two species.

Differences in structural characteristics between β-conglycinin and vicilin lead to unique surface properties and slightly different functional behavior. While both β-conglycinin and vicilin are N-glycosylated, the relative abundance of these groups, as well as their position on the molecular surface, differ between species (Kimura et al., 2008). Additionally, the α and α' subunits of β-conglycinin are distinct from those of vicilin as they contain highly acidic extension regions (Maruyama et al., 2002). These acidic extension regions, coupled with the position of N-linked glycans on the molecular surface of β-conglycinin, contributed to superior functional properties compared to vicilin. For example, Kimura et al. (2008) found β-conglycinin had a narrower insoluble pH range than vicilin, and that emulsions stabilized by the former had a smaller average particle size than those stabilized by the latter. On the other hand, both β-conglycinin and vicilin lack disulfide linkages, thus are often not considered good gelling agents, as discussed. However, Utsumi and Kinsella (1985) found β-conglycinin subunits

contributed to the network structure of gels by forming soluble complexes with the basic subunits of glycinin. An analogous relationship between vicilin subunits and legumin in thermally induced pea protein gels was not found in the literature.

Like β-conglycinin and vicilin, glycinin and legumin differ in their amino acid composition. Depending on the molecular isoforms present in each protein oligomer, the number of acidic and basic amino acids in glycinin and legumin may differ considerably (Kimura et al., 2008). The relative ratio of acidic and basic amino acids determines the net charge on the surface of the protein (Tang, Chen, & Ma, 2009). Surface net charge affects functional behavior, such as protein solubility, at specific pH and ionic strength (Gueguen, Chevalier, Schaeffer 1988; Kinsella, 1979). For example, when composed of the acidic group 2 subunits, glycinin exhibited a similar solubility profile to legumin at an ionic strength of 0.5 (Kimura et al., 2008). However, differences in their solubility profile were observed when glycinin's subunit composition differed or when the pH/ionic strength shifted (Kimura et al., 2008). As protein solubility is a critical prerequisite for other functional behaviors, such as emulsification or gelation, differences in solubility between glycinin and legumin will impact other functional properties.

Perhaps one of the most notable differences in the functional behavior between glycinin and legumin is found in their gelling properties. O'Kane et al. (2004c) found that 1.8% higher protein concentration was needed for legumin to form a gel compared to glycinin, and that the network structure of the former was considerably weaker than the latter. These observations may be attributed to inherent differences between glycinin and legumin. First, glycinin contains more cysteine residues (~8 per 60 kDa subunit) than legumin (~5 per 60 kDa subunit) (O'Kane et al., 2004c). Though gel formation is not

solely dependent on disulfide bond formation, fewer cysteine residues in legumin limit the extent of intermolecular association during network development (Sun and Arntfield, 2010, 2012). Consequently, legumin gels contain fewer network branches created by disulfide bonds and, therefore, have lower strength than those formed by glycinin at equal protein concentrations (O'Kane et al., 2004c). Beyond sulfhydryl groups and cysteine residues, hydrophobic residues have also been positively correlated to gel strength (Riblett et al., 2001). Therefore, glycinin's higher surface hydrophobicity and structural dependency on hydrophobic interactions may contribute to its superior gelling properties relative to legumin (Kimura et al., 2008). Lastly, exposed glycinin residues react at a slower rate than legumin residues during network formation (O'Kane et al., 2004c). As a result, the gel formed from glycinin is uniform, while that of legumin contains more irregular network strands (O'Kane et al., 2004c; Zheng, Matsumara, and Mori, 1991).

Overall, despite the similarities between the protein globulins of pea and soy, distinctions such as amino acid composition/sequence and inter-/intra- molecular interactions differentiate β-conglycinin from vicilin and glycinin from legumin. These inherent features contribute to unique functional behavior in the globulins of each species, which is a critical realization for replicating soy protein functionality using pea protein.

### 1.8 Compositional diversity in pea protein

The distribution of protein components in pea protein, namely 7S vicilin and 11S legumin, varies widely across pea cultivars. The 7S/11S ratio in pea protein ranges between 0.2-8.0 depending on factors such as agronomic practices, environmental

conditions, and most importantly, genetic origin (Casey et al., 1982; Mertens et al., 2012). These factors also affect the subunit amino acid composition and sequence of the individual pea globulins, which further contribute to heterogeneity in pea protein (Derbyshire et al., 1976). The influence of growing conditions and genetic variance on pea protein development, as well as their impacts on protein functionality and nutritional quality, will be discussed in further detail in the following sections.

### 1.8.1 Impact of agronomical and environmental conditions on pea protein development

The productivity of many crops is dependent on the culmination of climatic factors, environmental conditions, and agronomic practices (Mertens et al., 2012). While pea plants are most productive with 40-99 cm of annual precipitation and when grown between 13-18°C in well-drained, humus-rich soil with a pH of 6.5-7.0 (Tulbek et al., 2017), cultivation conditions outside of these parameters uniquely affect pea protein development. For example, water availability and its microelements, namely nitrogen, carbon, phosphorus, and potassium influence pea protein content (Mertens et al., 2012). According to Al-Karaki and Ereifej (1999) and Nikolopoulou, Grigorakis, Stasini, Alexis, and Iliadis (2007), drier years of cultivation enhanced pea protein development. Additionally, Nikolopoulou et al. (2007) found that cultivation in sandy soil caused lower total protein content in pea versus cultivation in loam soil.

Pea protein composition is also affected by environmental factors. Reports of the globulin and albumin content of pea protein ranged between 40-90% and 10-57%, respectively, depending on growth conditions (Mertens et al., 2012). Further, legumin

and vicilin are highly environmentally sensitive relative to other pea protein components (Bourgeois et al., 2009; Mertens et al., 2012), as they are quantitatively altered by factors such as temperature and nutrient availability during the cultivation period (Millerd et al., 1978; Mertens et al., 2012). Researchers also demonstrated that legumin is more vulnerable than vicilin to changes in growth environment, as legumin is not synthesized until late stages of the embryo's development (Chandler, Spencer, Randall, and Higgins 1984; Mertens et al., 2012). For example, pea seeds grown at 20°C consistently showed significantly lower legumin content than those grown at 25°C, whereas vicilin content was not significantly affected (Millerd et al., 1978). Moreover, under sulfur-deficient conditions, vicilin synthesis is maintained throughout development of the pea embryo, whereas legumin synthesis is greatly compromised or undetectable (Chandler et al., 1984).

As the global supply of yellow field pea is majorly sourced from Canada, Russia, the United States, France, and Australia, the inevitable variance in growing conditions across countries affects the physiological development of the pea seed and contributes to pea protein diversity, even within the same cultivar (Tulbek et al., 2017). However, some pea varieties may be more susceptible to environment effects than others (Casey et al., 1982).

### 1.8.2 Impact of genetic factors on pea protein components

Although environmental and agronomical factors certainly impact pea composition, genetic origin is the most significant cause of pea protein compositional diversity (Mertens et al., 2012). Genetic factors affect the assembly of acidic and basic

subunits in 11S and the amino acid composition and sequence, as well as the proteolytic cleavage sites, of all globulins (Gatehouse et al., 1981; Matta, Gatehouse, and Boulter, 1981; O'Kane et al., 2005; Tzitzikas et al., 2006). These variances cumulatively impact pea protein structure, functionality, and nutritional quality beyond the 7S/11S ratio. The heterogeneity that has been attributed to genetic effects will be discussed for legumin, vicilin, and convicilin.

There are reportedly ~8 different genes that encode legumin subunits (Domoney and Casey, 1985). According to Sun and Arntfield (2012), 4-5 distinct acidic polypeptides and 5-6 basic polypeptides have been identified in pea legumin based on differences in molecular weight, amino acid sequence, isoelectric point, and charge (Casey, 1979; Croy, Derbyshire, Krishna, and Boulter, 1979; Krishina, Croy, and Boulter, 1979; Thompson, Schroeder, and Dudman, 1978). Further, each subunit undergoes post-translational modification to varying degrees (Domoney and Casey, 1985; Gatehouse, Croy, Boulter, and Shewry, 1984). The variances among acidic and basic subunits impact the assembly of the legumin molecule, which contributes to its size and charge diversity among *Pisum* lines (Casey, 1979; Croy et al., 1979).

Genetic factors also contribute to heterogeneity within pea vicilin. There are several genes that control the mechanism of post-translational cleavage in vicilin subunits. Thus, depending on the gene expressed, polypeptide fragments of varying molecular weight and amino acid composition are produced during post-translational processing (Gatehouse et al., 1982; Gatehouse, Lycett, Delauney, Croy, and Boulter, 1983; Tzitzikas et al., 2006). Various fragments are assembled to form vicilin's monomers, which causes heterogeneity in vicilin's trimeric structure (Gatehouse et al.,

1981). Further, genetic factors determine the glycosylation site within vicilin subunits, which is an additional source of variation across cultivars (Gatehouse et al., 1981).

Lastly, despite limited studies, heterogeneity has been identified within pea convicilin. There have been contradicting reports of glycosylation among convicilin subunits. O'Kane et al. (2004b) found a glycosylated subunit of 14 kDa, while other researchers attested that convicilin subunits are not glycosylated (Boye et al., 2010; Newbigin et al., 1990; Tzitzikas et al., 2006). Further research uncovering the genetic factors impacting the structure of convicilin is needed.

### 1.9 Effects of pea protein diversity on nutritional quality

The amino acid composition of pea protein components is dependent on agricultural, environmental, and genetic influence (Casey et al., 1982; Gueguen and Barbot, 1988). For example, Wang and Daun (2004) found that the abundance of alanine, glycine, isoleucine, lysine, arginine, and threonine in pea protein differed depending on environmental conditions (Wang and Daun, 2004). Discrepancies in sulfur-containing amino acids have also been reported. Casey and Short (1981) found that legumin contained 2 cysteine and 3 methionine residues per ~60 kDa monomer, while Croy et al. (1980) reported 7 and 4, respectively. As the nutritional quality of pea protein is limited by the sulfur-containing amino acids, these discrepancies have a direct bearing on the nutritional quality of the protein (Wang and Daun, 2004). Accordingly, reports of PDCAAS for pea protein fluctuated between 0.54-0.89 among different cultivars (Mertens et al., 2012; Nosworthy and House, 2017; Rutherfurd et al., 2014).

### 1.10 Effects of pea protein diversity on functional behavior

While pea protein ingredients have amassed a significant share of the plant protein market, the significant diversity in protein profile across and within cultivars has notable implications on their functional behavior. Accordingly, substantial variation in solubility, emulsifying, gelling, and foaming properties of pea protein ingredients have been identified. For example, protein solubility ranged between 40-88% at pH 7 for pea protein isolates across different cultivars (Arteaga, Kraus, Schott, Muranyi, Schweiggert-Weisz, and Eisner, 2021; Barac et al., 2010; Boye et al. 2010; Fuhrmeister and Meuser, 2003; Stone, Avarmenko, Warkentin, and Nickerson, 2015a). O'Kane et al. (2005) isolated pea protein from 5 different cultivars and determined their gelling properties were cultivar specific. Arteaga et al. (2021) reported that emulsification capacity ranged from 600-835 mL/g protein across isolates sourced from 12 different pea cultivars, while Bu, Nayak, Bruggeman, Annor, and Ismail (2022) and Hansen et al. (2022) reported emulsification capacities as low as 341 and 441 mL/g protein, respectively. Emulsification activity index, emulsification stability index, and foaming capacity and stability were also dependent on cultivar (Cserhalmi, Czukor, and Gajzago-Schuster, 1998). For example, reports of foaming capacity fluctuated between 167.4 to 243.7%, while those of foaming stability ranged from 68 to 96% (Lam et al., 2018; Shevkani, Singh, Kaur, and Rana, 2015; Stone, Karalash, Tyler, Warkentin, and Nickerson, 2015b). As the quality of many food and beverage products is partially contingent on the consistent functional properties of protein ingredients, these discrepancies in pea protein functionality have consequently limited the widespread use of pea protein ingredients in food applications.

## 1.11 Targeted breeding as an approach to enhance pea protein functionality and nutritional quality

The vast diversity in pea protein contributes to inconsistent functional behavior and nutritional quality. Though many efforts have been made by researchers to enhance pea protein functionality following several approaches, including novel extraction and processing strategies (Hansen, et al., 2022; Stone et al., 2015b), cold plasma modification (Bu et al., 2022, 2023), Maillard-induced glycation (Kutzli et al., 2020; Schneider, Bu, and Ismail, 2023; Zhao et al., 2022), enzymatic hydrolysis (Arteaga et al., 2022; Barac et al., 2012), and physical modifications (Mirmoghtadaie, Shojaee Aliabadi, and Hosseini, 2016; Pedrosa and Ferreira, 1994), these strategies do not address the intrinsic characteristics of pea protein. Thus, a more sustainable solution, such as targeted breeding, is needed to address the lower and inconsistent functionality and nutritional quality of pea protein relative to soy protein.

Traditionally, whether crops are destined for either human food or animal feed, the main priorities for breeding programs are to produce cultivars that produce high yield, reach maturation earlier, and are resistant to lodging and disease (Lam et al., 2018). This has also been the case for field pea over its history of cultivation (Vera, 1999). However, many researchers have alluded to the potential significance of breeding programs to address the inconsistencies of pea protein and improve its application in the food industry. For example, Casey and Short (1981) and Millerd et al. (1978) proposed that increasing the proportion of legumin to vicilin would be a desirable objective of breeding programs to optimize nutritional quality of pea protein. However, Barac et al. (2010) reported that a higher abundance of vicilin relative to legumin enhanced the extractability

of the protein. Tzitikas et al. (2006), on the other hand, proposed that there should be breeding efforts to reduce convicilin content given its potential negative impacts on pea protein functionality.

Though the targeted outcomes for pea breeding programs could vary, stabilizing and optimizing the protein composition of yellow field pea through genetic factors should be prioritized. Achieving a consistent 7S/11S ratio in pea protein would be an ideal outcome for targeted breeding programs, since this ratio is related to both the nutritional value and functional characteristics of protein-enriched products (Barac et al., 2010; Gueguen and Barbot, 1988; Tzitizkas et al., 2006). For example, a correlation between 7S/11S ratio and functionality has been demonstrated in soy protein, in which the ratio was useful in predicting the behavior of proteins during extrusion processing (Wu, Hua, Chen, Kong, and Zhang, 2017, tofu firmness and yield (Mujoo, Trinh, and Ng, 2003), and interfacial properties such as foaming capacity and stability (Zhu et al., 2020). Establishing a similar correlation between 7S/11S ratio in pea protein and functional properties and nutritional quality would be invaluable to identifying the optimum protein profile for targeted cultivar selection.

### 1.12 Previous research investigating the impact of 7S/11S ratio on pea protein functionality

The variation in 7S/11S ratio across and within pea cultivars is a major contributor to the diverse functional and nutritional properties of pea protein (Barac et al., 2010; Tzitzikas et al., 2006). As discussed, establishing a correlation between 7S/11S ratio in pea protein and resultant functional properties would be invaluable to identifying

the optimum protein profile for certain applications in the food industry and for targeted breeding programs. This has inspired various research efforts to date to investigate the effects of pea protein profile variance on functionality by isolating 7S vicilin and/or 11S legumin from a specific pea cultivar, mixing them in varying ratios, and evaluating certain structural and functional properties. Although such efforts considered only a single pea variety to reduce potential cross-cultivar effects on functional properties, contradictory functional properties of 7S vicilin, 11S legumin, and their ratios were nevertheless reported.

For example, inconsistencies in the emulsifying behavior of pea protein components were found. Dagorn-Scaviner, Gueguen, and Lefebvre (1986, 1987) mixed pea 7S vicilin and 11S legumin in four different 7S/11S ratios (0.33, 0.5, 1.0, and 3.0) and evaluated surface activity and emulsifying properties. They determined that higher abundance of 7S vicilin enhanced the emulsification capacity, emulsion stability, and emulsifying activity index of pea protein isolates. However, they found that the globulin composition of the isolates did not completely explain their emulsifying behaviors. Meanwhile, Koyoro and Powers (1987) evaluated the emulsification capacity of 7S vicilin, 11S legumin, and one 7S/11S mixture of unspecified ratio. Their results, in contrast to Dagorn-Scaviner et al. (1986, 1987), were that 11S legumin had higher emulsification capacity than both 7S vicilin alone and the 7S/11S mixture.

Reports of the gelling behavior of pea protein components have also been contradictory. Bora, Brekke, and Powers (1994) evaluated the gelling properties of crude and purified 7S vicilin fractions, 11S legumin fractions, and a mixture with a 7S/11S ratio of 1.8. The authors found that in both the crude and purified samples, gel hardness

increased as the proportion of 11S legumin decreased. Instead, stronger protein gels formed from pea protein samples containing a higher abundance of vicilin. In contrast, O'Kane et al. (2004a) prepared three 7S/11S ratios (0.22, 0.57, and 1.2) from purified 7S vicilin and 11S legumin fractions and found that legumin formed thermally induced gels, while certain vicilin subunits inhibited gelation.

Because of these contradictory findings, the impact of pea protein profile on functional behavior remains unclear. Additionally, these studies are limited in that they only evaluated a few structural and functional properties of 7S vicilin, 11S legumin, and their ratios. Furthermore, the current understanding of the functionality of11S legumin has largely been acquired based on its isolated and purified form. No studies have evaluated the functional effects of enriching a pea protein isolate with 11S legumin. This research is necessary to understand how the inherent components and environmental factors introduced by the original pea protein matrix affect protein-protein interactions and resultant functional behavior of 11S legumin in varying abundance. To expand on the current research landscape and broaden the understanding of the factors that influence pea protein functionality, a holistic investigation of protein structural, functional, and nutritional properties of 7S vicilin, 11S legumin, and multiple ratios is necessary.

### 1.13 Conclusions

Pea protein is one of the fastest growing plant proteins in global alternative product launches, attributed to the agronomic benefits of growing pea, a low production cost, and its current non-allergenic, non-genetically modified status. As consumers continue to seek alternatives for soy protein, these advantages of pea protein

demonstrated the opportunity for industry to incorporate pea protein in plant-based foods and beverages in lieu of soy protein. However, despite having considerable homology to soy protein, pea protein has inferior functionality and nutritional quality in food and beverage applications. Further, a wide variability in the functional behavior and nutritional quality of pea protein, both within and across pea cultivars, has been identified. The functional inferiority and inconsistency in pea protein relative to soy protein has been attributed to the inherently lower abundance of 11S in pea protein and its wide diversity in 7S/11S ratio, respectively. Agronomic factors, environmental conditions, and genetic factors have been found to contribute to this heterogeneity in pea protein. Thus, pea protein's lesser, unpredictable functional behavior, coupled with its inherently lower nutritional quality relative to soy protein, presents notable formulation and consistency challenges to the food industry. As a result, the widespread acceptance of pea protein by both industry and consumers has been jeopardized.

To improve the quality and consistency of pea protein ingredients, a foundational knowledge relating protein profile to functional behavior and nutritional quality in food and beverage applications is critical. Though the relationship between soy protein components and resultant functionality and nutritional quality has been well documented, the impact of pea protein profile on functional behavior and nutritional quality remains unclear due to contradictory findings in the literature. Therefore, a holistic investigation of the impact of 7S/11S ratio on pea protein structure, functionality, and nutritional quality is necessary. Furthermore, the lower understanding of the inherent molecular properties of pea 11S legumin compared to soy 11S glycinin, coupled with the former's wide compositional diversity both within and among cultivars, necessitates further study

of its structure and functional behavior both isolated and within the original pea protein matrix. This type of research is necessary to understand how the inherent components and environmental factors introduced by the pea protein matrix affect protein-protein interactions and resultant functional behavior of 11S legumin in varying abundance. Lastly, the intrinsic variance in amino acid composition of 7S vicilin and 11S legumin necessitates additional evidence relating pea protein profile with nutritional quality to address the lower and inconsistent nutritional quality of pea protein compared to soy protein.

Ultimately, establishing a correlation between 7S/11S ratio of pea protein and its functionality and nutritional quality will contribute to the needed knowledge and guidance for future studies aiming to predict pea protein behavior based on its protein profile. Outcomes of a thorough investigation may also be used to devise strategies for improving the quality and consistency of pea protein ingredients, such as targeted breeding programs. Such synergistic efforts would have a monumental impact on the continual development of pea as a source of functional and nutritious protein for the global food supply.

# Chapter 2: Materials and Methods

## 2.1 Materials

Yellow pea flour was provided by AGT Foods (Regina, SK, Canada). Commercial pea protein isolate (cPPI, ProFam® Pea 580, 79.5% protein) and commercial soy protein isolate (cSPI, ProFam® 974, 90.7% protein) were provided by Archer Daniels Midland (ADM) (Decatur, IL, USA). When not in use, samples were stored at -20°C. Criterion™ TGX™ 4-20% precast gels, Laemmli sample buffer, 10X Tris/Glycine/sodium dodecyl sulfate (SDS) running buffer, Imperial™ Protein Stain, and Precision Plus Protein™ molecular weight (MW) marker were purchased from Bio-Rad Laboratories, Inc. (Hercules, CA, USA). A Superdex™ 200 Increase 10/300 GL Prepacked Tricorn™ Column, gel filtration low molecular weight (LMW) calibration kit, and gel filtration high molecular weight (HMW) calibration kit for size-exclusion high performance liquid chromatography (SE-HPLC) were purchased from Cytiva (Marlborough, MA, USA). A BioSuite DEAE AXC, 1000Å column for anion exchange high performance liquid chromatography (AXC-HPLC) was purchased from Waters Corporation (Milford, MA, USA). For amino acid analysis, a Waters Acquity ultra performance liquid chromatography ethylene bridged hybrid (UPLC-BEH) C18 column (ACCQ-TAG ULTRA C18 100), AccQ Tag Ultra eluents A and B, 6-aminoquinolyl-*N*-hydroxysuccinimidylcarbamate (AQC), and Amino Acid Standard H mixture were provided by Waters (Milford, MA, USA). L-Tryptophan (Trp) and L-Norvaline (Nval) were purchased from Sigma-Aldrich (St. Louis, MO, USA). A Protein Digestibility Assay Kit (K-PDCAAS) was purchased from Megazyme International Co. (Bray, Ireland).

## 2.2 Production of pea protein isolate (PPI)

Native pea protein isolate (nPPI) was extracted and purified following the pH extraction method described by Hansen, Bu, & Ismail (2022). The Dumas method (AOAC 990.03) was used to determine the protein purity of nPPI (86%), using a LECO® FP828 nitrogen analyzer (LECO, St. Joseph, MI, USA) and a conversion factor of 6.25.

## 2.3 Production of 7S vicilin and 11S legumin enriched fractions

Commercial pea flour was fractionated into 7S vicilin and 11S legumin enriched fractions following a procedure described by Suchkov, Popello, Grinberg, & Tolstoguzov (1990) with modifications. Pea flour was fully dispersed in a tenfold volume of double distilled water (DDW) and adjusted to pH 8.0 with 2 N NaOH. The suspension was stirred for 1 hour at 50°C and then centrifuged (5000 x g, 30 minutes) to separate insoluble materials. Sodium chloride (NaCl) was added to the supernatant to reach 0.5 M, and the solution was stirred until NaCl was completely dissolved. The pH was adjusted to 4.8 using 2 N HCl, and the suspension was centrifuged (5000 x g, 30 minutes) to isolate globulin proteins. The supernatant was diluted with DDW to 0.3 M NaCl and centrifuged (1000 x g, 10 minutes) to separate 7S vicilin (supernatant) from 11S legumin (pellet). The 7S vicilin-containing supernatant was cooled to 5°C and maintained at this temperature overnight. The solution was centrifuged (1000 x g, 15 minutes, 5°C), and the supernatant was diluted to 0.15 M NaCl with 5°C DDW. The solution was centrifuged (1000 x g, 15 minutes), and the precipitate collected as the 7S vicilin enriched fraction, which was suspended in DDW (1:5 w/v), neutralized, dialyzed, and lyophilized. The 11S legumin-containing pellet was fully dispersed in a tenfold volume of 0.6 M NaCl and centrifuged

(5000 x g, 30 minutes). The supernatant was diluted to 0.3 M NaCl with DDW and left overnight at room temperature. The precipitate formed was collected as the 11S legumin enriched fraction and then suspended in DDW (1:5 w/v), neutralized, dialyzed, and lyophilized. The protein content of the enriched fractions (7S vicilin: 100%; 11S legumin: 100%) was determined by the Dumas method with a conversion factor of 6.25, and the samples were stored at -20°C.

## 2.4 Evaluation of the effectiveness of 7S vicilin and 11S legumin fractionation

### 2.4.1 Protein profiling by SDS-PAGE

The protein subunit distribution in the 7S vicilin and 11S legumin enriched fractions was visualized using SDS polyacrylamide gel electrophoresis (SDS-PAGE), under reducing and non-reducing conditions, as described by Boyle, Hansen, Hinnenkamp, & Ismail (2018). All samples were loaded at equal protein amount (50 $\mu$g protein in 5 µL). The protein profile of each enriched fraction was compared to previous reports for pea vicilin and pea legumin subunit distribution (Casey & Domoney, 1999; Matta, Gatehouse, & Boulter, 1981) to verify the efficiency of 7S vicilin and 11S legumin fractionation.

### 2.4.2 Size-exclusion high performance liquid chromatography (SE-HPLC)

The protein components in the enriched fractions were also evaluated by size-exclusion high performance chromatography (SE-HPLC). A Shimadzu HPLC system (Shimadzu Scientific Instruments, Colombia, MD, USA) equipped with Superdex 200 Increase 10/300 GL Tricorn™ (10x300 mm) column, SIL-10AF auto injector, LC-20AT

pump system, CTO-20A column oven, SPD-M20A photo diode array detector, and a CBM-20A communication module was used to separate proteins based on molecular weight. The analysis was performed following the method reported by Bruckner-Guhmann, Heiden-Hecht, Sozer, & Drusch (2018) and modified by Bu, Nayak, Bruggeman, Annor, & Ismail (2022). Samples (1% protein concentration, w/v) were solubilized in pH 7 phosphate buffer (0.05 M sodium phosphate with 0.1 M sodium chloride) under magnetic agitation (250 rpm) for 2 hours at room temperature. Samples were passed through a 0.45 μm filter, injected (100 μL), and separated isocratically using pH 7 phosphate buffer mobile phase at a flow rate of 0.5 mL per minute for a total run time of 60 min. Detection and analysis were performed at 280 nm. Peak identities were assigned based on reported molecular weights (Barac et al., 2010; Gatehouse, Lycett, Croy, & Boulter, 1982; Tzitzikas et al., 2006).

### 2.4.3 Anion exchange high performance chromatography (AXC-HPLC)

To further evaluate their protein constituents, the enriched fractions were subjected to weak AXC-HPLC using the same Shimadzu system described in section 2.4.2 but equipped with a Waters - BioSuite DEAE AXC, 1000Å, 10 $\mu$m 7.5x75 mm column. Proteins were separated based on the method described by Gueguen, Vu, & Schaeffer (1984), with modifications. Samples (5% protein concentration, w/v) were solubilized in pH 7 phosphate-citrate (0.16 M) under magnetic agitation (250 rpm) for 2 hours at room temperature. Samples were passed through a 0.45 μm filter, injected (100 μL), and separated following a gradient elution at a flow rate of 0.35 mL/min. From 0 to 5 min the mobile phase was held at 0 M NaCl, followed by a linear increase to 0.5 M

NaCl from 5 to 10 min, then held at 0.5 M NaCl from 10 to 15 min, followed by a linear decrease to 0 M NaCl from 15 to 20 min, and finally column equilibration at 0 M NaCl from 20 to 40 min. Detection and analysis were performed at 280 nm. Peak identities were assigned based on Gueguen et al. (1984).

## 2.5 Production of protein isolates with differing 7S vicilin to 11S legumin ratios

### 2.5.1 Reconstituted protein isolates

Aliquots of the 7S vicilin and 11S legumin enriched fractions were coded as 100V and 100L, respectively, and reserved for analysis. The letter "V" (vicilin) represented the 7S vicilin enriched fraction, and the letter "L" (legumin) represented the 11S legumin enriched fraction. The remaining enriched fractions were then blended with mortar and pestle to generate three samples with differing protein ratios: 80V-20L, 50V-50L, and 20V-80L. Each sample was coded relative to its % composition of enriched fractions on a weight basis.

### 2.5.2 Legumin-enriched nPPI

To estimate the 7S/11S ratio in nPPI, its protein profile was evaluated, in triplicate, by SDS-PAGE/densitometry. SDS-PAGE was performed following the method described in section 2.4.1. The different bands under non-reducing conditions were assigned to legumin, vicilin, and convicilin according to their molecular weights, and their respective intensities were determined (Tzitzikas et al., 2006) using the Molecular Imager Gel Doc XR system (Bio-Rad Laboratories) with Quantity One software (version 4.6.7). The 7S/11S ratio was determined using only the bands associated with legumin

and vicilin; convicilin was not included in the ratio (Lam, Warkentin, Tyler, & Nickerson, 2017). nPPI was determined to have a 7S vicilin to 11S legumin ratio of 80:20 (data not shown). Therefore, nPPI-50LE and nPPI-80LE were produced by addition of the 11S legumin enriched fraction directly to nPPI to achieve 50% and 80% 11S legumin compositions (w/w), respectively. A sample calculation is given in *Appendix A*. These samples were produced to evaluate the impact of higher 11S legumin ratio within the nPPI matrix, in comparison to the reconstituted isolates, on the overall structure and functionality.

### 2.5.3 Reconstituted protein isolates with increased salt content

100V-salt and 100L-salt were produced by the addition of NaCl to 100V and 100L, respectively, to determine the effect of increased ionic strength (0.5μ) on the structure and function of the protein fractions. First, the ionic strength of a 0.5M NaCl solution was determined to be 0.5 (*Appendix B*). Next, the initial salt content of each sample was calculated based on its ash content (as determined by dry ashing, AOAC 942.05) with the assumption that most of the salt present was residual NaCl. This assumption was made due to the use of NaCl solutions to fractionate the 7S vicilin and 11S legumin proteins, and NaCl production due to pH adjustments with NaOH and HCl (Kornet et al., 2021). Aliquots of 100V and 100L were fully dispersed in DDW, and NaCl was added to reach 0.5μ (*Appendix C*). Samples were stirred at room temperature for 4 hours and lyophilized.

## 2.6 Protein structural characterization

### 2.6.1 Protein profiling and molecular weight distribution by SDS-PAGE and SE-HPLC

The protein profile of all samples except 100V-salt and 100L-salt was determined by SDS-PAGE as described in section 2.4.1. The same HPLC system and method described in section 2.4.2 was performed to determine molecular weight distribution, with a modified run time of 85 min. Molecular weights were calculated by running gel filtration calibration standards (HMW and LMW kits). Sample chromatograms and a calibration curve are shown in *Figures 5 & 6, Appendix D*. Relative peak areas (the ratio of the area of a single peak to total peak area for a sample) were used to monitor differences in molecular weight distribution among the samples.

### 2.6.2 Protein denaturation as determined by differential scanning calorimetry (DSC)

The denaturation temperature and enthalpy of the protein fractions and isolates were analyzed in triplicate, using a Mettler DSC (Mettler Toledo, Columbus, OH, USA), following the method outlined by Tang, Choi, & Ma (2007) and modified by Bu et al. (2022). Endothermic peaks were integrated for each replicate using the Mettler Toledo STARe Software version 11.00.

### 2.6.3 Protein surface properties

The spectrofluorometric method reported by Boyle et al. (2018) and modified by Bu et al. (2022) was utilized to measure, in triplicate, the surface hydrophobicity of the

protein samples. A sample calculation and plot (*Figure 7*) is shown in *Appendix E*. A dynamic light scattering instrument (Malvern Nano Z-S Zetasizer) was used to measure, in triplicate, the zeta potential of protein samples as an indication of surface charge.

### 2.6.4 Attenuated total reflectance Fourier transform infrared spectroscopy (ATR-FTIR)

Protein samples were analyzed by a Fourier transform infrared spectrometer (Thermo Scientific™ Nicolet™ iS50 FTIR) (Bu et al., 2022, 2023), and OMNIC® software was used to translate the ATR-FTIR spectra to transmission spectra. The major peaks in the amide I region (1600 $cm^{-1}$ –1700 $cm^{-1}$) were identified by the normalized second derivative using the embedded function in GraphPad (Prism 8). The secondary structures (α-helix, β-sheet, β-turn, and random coil) were assigned according to Sadat and Joye (2020) and Housmans et al. (2022).

## 2.7 Protein functional characterization

### 2.7.1 Protein solubility

The protein solubility of the samples, prepared at 5% protein concertation (w/v), was measured, in triplicate, following the procedure described by Wang & Ismail (2012), at pH 3.4 and pH 7, with and without heating at 80°C for 30 min. Protein solubility was taken as the proportion of soluble protein to the total protein present in the initial solution as determined by the Dumas method. Protein solubility was expressed as the percentage

of soluble protein (present in the supernatant) compared to the total protein (present in the initial sample). An example calculation is shown in *Appendix F*.

### 2.7.2 Gel strength

Thermally induced gels (15 or 20% protein concentration, w/v, 95°C for 30 minutes) were prepared, in triplicate, as outlined by Bu et al. (2022). Samples were cooled to room temperature and a TA-TX Plus Texture Analyzer (Stable Micro Systems LTD, Surrey, UK) equipped with a 100 mm diameter probe with a test speed of 1 mm $s^{-1}$ and distance of 0.5 mm from the plate was used to rupture the gel. The force (N) required for rupture was reported as gel strength.

### 2.7.3 Emulsification capacity

The emulsification capacity (EC) of protein samples (1 and 2% protein concentration, w/v) was determined, in triplicate, following the method outlined by Boyle et al. (2018) and modified by Hinnenkamp & Ismail (2021). EC, expressed as g of oil emulsified per 1 g of protein, was calculated. An example calculation is shown in *Appendix G*.

## 2.8 Protein Digestibility – Corrected Amino Acid Score (PDCAAS)

### 2.8.1 Amino acid analysis

The amino acid profile of all PPIs, except 100V-salt and 100L-salt, was determined in duplicate. Cysteine and methionine were quantified following AOAC 994.12, while the remaining amino acid profile was determined following the acidic and

alkaline digestion methods outlined by Temtrirath (2022) and La Cour, Jorgensen, & Schjoerring (2019), respectively, with modifications. Samples were subjected to acid digestion in a microwave digestion unit (Mars 6, CEM, NC, USA) at 155 °C for 15 min, and to alkaline digestion in a preheated conventional oven at 110 °C for 20 h. NVal (50 mM) was used as the internal standard (IS) for both acid and base digestion. Samples were then subjected to a pre-column derivatization using AQC at 55°C for 10 min as outlined by Temtrirath (2022). Amino Acid Standard H mixture (1-100 pmol) was simultaneously prepared and derivatized following manufacturer instructions. Amino acid composition was determined following the methods of Ma et al. (2018) and Temtrirath (2022) using a Waters ACQUITY UPLC H-class system (Waters Corporation, Milford, MA, USA) equipped with a Waters Acquity UPLC-BEH C18 column (100 mm× 2.1 mm, with 1.7 $\mu$m particle size), a quaternary solvent manager (QSM), a sample manager with a Flow-Through Needle (FTN), a column oven (CH-A), and a photodiode array (PDA) detector. Amino acid peaks were integrated at 260 nm, identified based on the retention times of the corresponding standards, and quantified based on calibration curves. Integration and data processing were done using Empower 3 Software (Waters Corporation, Milford, MA, USA). A sample chromatogram of the standard and a sample calibration curve of an amino acid are shown in *Figures 8 & 9, Appendix H*. The amino acid composition of the samples is shown in *Table 5 (Appendix I)*.

### 2.8.2 Protein digestibility

*In vitro* protein digestibility of pea protein samples, except 100V-salt and 100L-salt, was determined in duplicate using the K-PDCAAS Megazyme kit and the provided

instructions. The amino acid score (AAS) and PDCAAS were then calculated according to the reference amino acid pattern is that required for children (6 months to 3 years) as defined by FAO/WHO Expert Consultation (1991). Example calculations are provided in *Appendix I***.**

### 2.9 Statistical analysis

IBM SPSS Statistics software version 27.0 for Windows (SPSS Inc., Chicago, IL, USA) was used to perform analysis of variance (ANOVA) and t-tests. Tukey-Kramer Honest Significant Difference (HSD) multiple means comparison test was used to determine significant differences ($P \leq 0.05$) among the means (*Tables 7 – 35, Appendix J)*. Two-sample, unpaired t-test was used to determine significant differences ($P \leq 0.05$) between the means of two different samples.

## Chapter 3: Results and Discussion

### 3.1 Effectiveness of 7S vicilin and 11S legumin fractionation

#### 3.1.1 Protein profile of isolated fractions

The protein profiles of 100V and 100L were visualized by SDS-PAGE (*Figure 1 A and 1B, lanes 4 and 10*) to assess the effectiveness of the employed fractionation. In 100V, protein bands corresponding to convicilin (~70 kDa), vicilin (~47 kDa), and vicilin fragments (~30-36 kDa, ~15-19 kDa), as identified based on previous reports (Tzitzikas et al., 2006), were noted under non-reducing and reducing conditions (*Figure 1, lane 4)*. A couple of faint bands corresponding to large molecular weight polymers (~150-200

kDa) were observed under non-reducing conditions, but were not apparent under reducing conditions, indicating involvement of disulfide linkages. These polymers could potentially be residual legumin, as the shifts in pH levels and ionic strengths during fractionation might have caused residual legumin to dissociate/associate into a mixture of trimers and/or dimers (Barac, Pesic, Stanojevic, Kostic, & Cabrilo, 2015).

The bands corresponding to vicilin were darker in intensity in 100V compared to counterparts in nPPI (*Figure 1, lanes 3-4*). On the other hand, individual legumin bands (~60 kDa under non-reducing conditions, ~40 and ~20 kDa under reducing conditions; Tzitzikas et al., 2006) were not visible in 100V (*Figure 1A and 1B, lane 4*). These observations confirmed that 100V was enriched with 7S vicilin and had negligible 11S legumin contamination.

Meanwhile, 100L had prominent legumin bands, much darker in intensity than their counterparts in nPPI (*Figure 1A and 1B, lanes 3 and 10*). This observation confirmed that 100L was mostly comprised of 11S legumin. A greater abundance of large molecular weight polymers, as indicated by dark smearing in the upper region of the lane, were noted in 100L compared to 100V (*Figure 1A, lane 10 compared to lane 4*). Presence of these polymers was likely induced by the additional changes in extraction conditions to isolate the 11S legumin fraction, as discussed. Changes in pH, temperature, and ionic strength can alter protein conformation by disrupting electrostatic and hydrophobic forces, inducing denaturation and subsequent polymerization (Damodaran & Parkin, 2017). Under reducing conditions, smearing in the upper region of 100L's lane was reduced yet was still apparent, indicating the involvement of covalent bonds beyond disulfide linkages (*Figure 1B, lane 10)*.

Protein bands corresponding to convicilin and vicilin were also observed in 100L (*Figure 1A and 1B, lane 10)*. Contamination of convicilin and various vicilin subunits in an isolated legumin fraction has been previously reported (Bora et al., 1994; Koyoro & Powers, 1987; Mession, Assifaoui, Cayot, & Saurel, 2012; Mession, Chihi, Sok, & Saurel, 2015; O'Kane et al., 2004a).

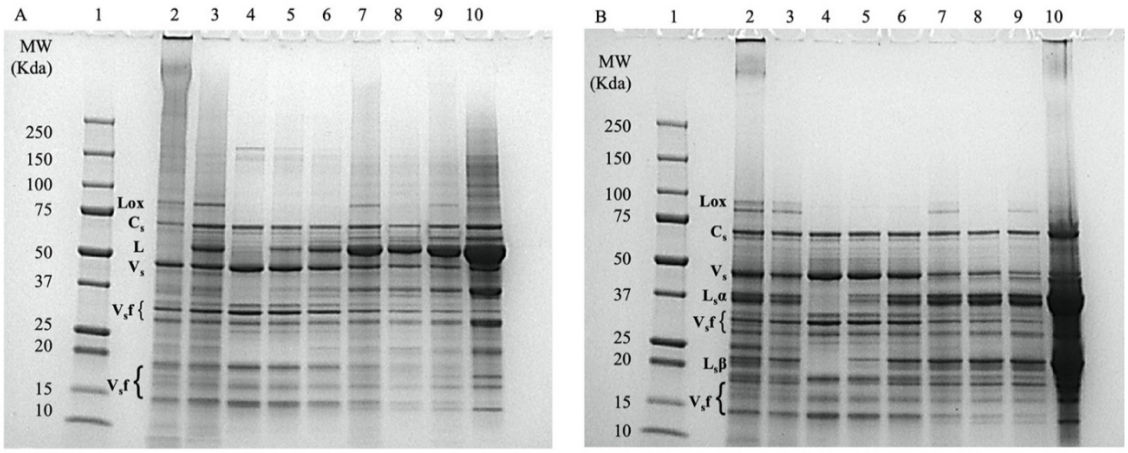


**Figure 1.** SDS-PAGE gel visualization of the protein profiles of the different protein fractions and mixtures under (A) non-reducing and (B) reducing conditions. Lane 1: molecular weight standard; lane 2: cPPI; lane 3: nPPI; lane 4: 100V; lane 5: 80V-20L; lane 6: 50V-50L; lane 7: nPPI-50LE; lane 8: 20V-80L; lane 9: nPPI-80LE; lane 10: 100L. Lox: lipoxygenase; Cs: subunits of convicilin; Vs: subunits of vicilin; Lsα: acidic peptides cleaved from legumin subunits; Lsβ: basic peptide cleavage from legumin subunit; Vsf: fractions of vicilin subunits result from post-translational cleavages.

### 3.1.2 Purity of the protein fractions

To further determine the effectiveness of fractionation, the purity of the 7S vicilin and 11S legumin enriched fractions was evaluated by AXC and SE-HPLC (*Figure 2*). Following AXC, chromatographic peaks corresponding to vicilin, convicilin, and legumin were identified within the 7S vicilin enriched fraction (*Figure 2A*) based on the elution patterns reported by Gueguen et al. (1984). The sensitivity of the UV detection confirmed the presence of residual 11S legumin within the 7S vicilin enriched fraction,

while protein bands corresponding to legumin were not visible by SDS-PAGE (*Figure 1*). The combined observations indicated that the 11S legumin contamination might not have been quantitatively significant. Meanwhile, one prominent, high intensity chromatographic peak corresponding to legumin was identified within the 11S legumin enriched fraction (*Figure 2B*). A minor/low intensity peak was identified as residual convicilin (Gueguen et al., 1984), corroborating the presence of its corresponding protein band (*Figure 1*).

Similarly, three chromatographic peaks (*Figure 2C*) were identified within the 7S vicilin enriched fraction by SE-HPLC as legumin, convicilin, and vicilin, based on previously reported elution patterns (Gatehouse et al., 1982). The legumin peak had a relatively low intensity compared to that of vicilin, confirming the residual presence of 11S legumin in the 7S vicilin enriched fraction, as discussed. On the other hand, a high intensity chromatographic peak corresponding to legumin was identified in the 11S legumin enriched fraction (*Figure 2D*). Another low-intensity chromatographic peak was also noted in the 11S legumin enriched fraction, potentially corresponding to dimers and/or trimers of legumin. While both AXC and SE-HPLC revealed contamination in the 7S vicilin and 11S legumin enriched, residual counterparts were present in relatively low proportions. Although similar findings prompted some researchers to use chromatography to further purify the crude fractions, purification had very low yield and was time consuming, limiting the extent of structural and functional characterization of the isolates (Bora et al., 1994; Koyoro & Powers, 1987; O'Kane et al., 2004a). Further, Bora et al. (1994) reported there was no functional difference between crude and purified legumin fractions. Therefore, in this study, characterization of crude fractions, rather than

purified ones, was chosen to fill knowledge gaps regarding pea 7S vicilin, 11S legumin, and their ratios.

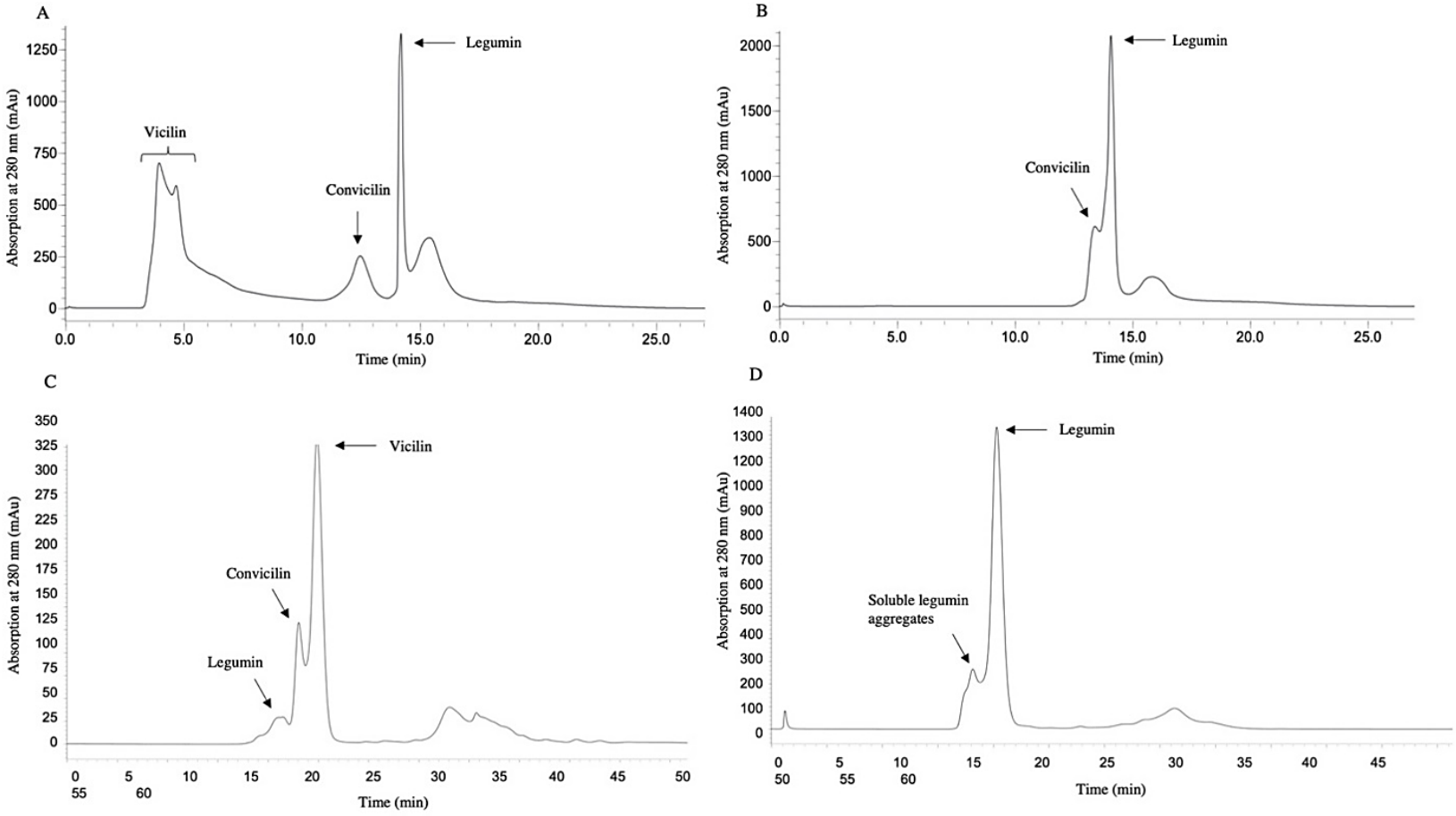


**Figure 2.** Visualization of the distribution of protein components in the enriched fractions by anion-exchange chromatography (AXC) and size-exclusion chromatography (SEC). Chromatogram (A) 7S vicilin enriched fraction under AXC, (B) 11S legumin enriched fraction under AXC, (C) 7S vicilin enriched fraction under SEC, (D) 11S legumin enriched fraction under SEC.

## 3.2 Structural properties of the isolated fractions, reconstituted protein isolates, and legumin-enriched nPPIs

### 3.2.1 Protein profile and molecular weight distribution

The protein profiles of the reconstituted protein isolates and legumin-enriched nPPIs were consistent with the targeted 7S/11S ratios (*Figure 1*). Under non-reducing conditions, the intensity of the legumin band (~60 kDa) increased following its increasing abundance across 80V-20L, 50V-50L, 20V-80L, and 100L *(Figure 1, lanes 5-10)*. Meanwhile, the intensity of vicilin bands (~45, 30-36, 15-19 kDa) progressively decreased across 100V, 80V-20L, 50V-50L, 20V-80L, and 100L, which affirmed decreasing vicilin abundance. These legumin and vicilin patterns were similar among the samples under reducing conditions, though the former was exhibited in its subunits (~40 kDa and 20 kDa) rather than in the monomer form (~60 kDa).

Dark smearing in the upper portion of the lanes was especially evident in cPPI, nPPI, and 100L under non-reducing conditions (*Figure 1A, lanes 2, 3, 10*). Such smearing was also noted, albeit less intense, in nPPI-50LE and nPPI-80LE (*Figure 1A, lanes 7 and 9*). 100V, 80V-20L, 50V-50L, and 20V-80L exhibited the least smearing among the samples (*Figure 1A, lanes 4, 5, 6, 8*). Under reducing conditions, the noted smearing was reduced (*Figure 1B*), which indicated that all the mentioned isolates contained disulfide-linked polymers to certain extents. However, residual smearing was noted in cPPI and 100L lanes under reducing conditions, which indicated protein polymerization via covalent bonding beyond disulfide linkages (*Figure 1B, lanes 2 and 10*). The extent of polymerization is mostly attributed to the extraction process. The use

of harsh extraction parameters to produce cPPI induced protein denaturation and subsequent polymerization (Hansen et al., 2022). Meanwhile, the fractionation conditions utilized in this study induced greater polymerization in the 11S legumin enriched fraction than in the 7S vicilin enriched fraction, mostly due to protein denaturation.

To further characterize the molecular weight (MW) distribution of soluble aggregates, functional proteins (legumin, vicilin, and convicilin), and low molecular weight polypeptides, samples were analyzed by SE-HPLC following solubilization in phosphate buffer, phosphate buffer + SDS, and phosphate buffer + SDS and BME (*Table 1, Figure 3*). The chromatographic peaks corresponding to >450 kDa polymers were collectively identified as soluble aggregates, since they were soluble in the sample buffer and passed filtration (0.45 $\mu$m) prior to injection on the column. Chromatographic peaks corresponding to hexameric legumin, trimeric convicilin, and trimeric vicilin were identified according to their reported MW ranges (Barac et al., 2010; Gatehouse et al., 1982; Tzitzikas et al., 2006).

In phosphate buffer, cPPI had a high relative abundance of soluble aggregates but significantly ($P < 0.05$) the least functional proteins among all samples (*Table 1, Figure 3A*), which complimented the SDS-PAGE observation (*Figure 1*). This MW distribution indicated that most functional proteins in cPPI likely polymerized into large insoluble aggregates that did not pass through the filter (0.45 μm) and thus were not represented by a chromatographic peak. In contrast, nPPI had a significantly lower abundance of soluble aggregates than cPPI but a significantly higher percent distribution of each functional protein (*Table 1*). Among the reconstituted protein isolates, the abundance of soluble aggregates significantly increased with the relative proportion of 11S legumin (*Table 1*).

Similarly, the relative abundance of soluble aggregates was significantly higher in nPPI-80LE than nPPI-50LE. This observation complemented that of the SDS-PAGE, where protein bands corresponding to high molecular weight polymers increased in intensity with higher proportion of the 11S legumin fraction. The difference in soluble aggregates among all the samples was attributed to the effects of fractionation conditions on protein structure, as discussed.

Meanwhile, the MW distribution of functional proteins in the isolated fractions, reconstituted isolates, and legumin-enriched nPPIs was consistent with the targeted 7S/11S ratios (*Table 1*). Relative abundance of legumin was significantly the highest in 100L and decreased following its lower abundance across nPPI-80LE, 20V-80L, nPPI-50LE, 50V-50L, 80V-20L, and 100V (*Table 1*). On the other hand, the relative abundance of vicilin was significantly the highest in 100V and decreased with the reduction in 7S vicilin proportion in the different samples. Convicilin was present in similar percent relative abundance in all the samples, which affirmed the contamination noted in the legumin-enriched fraction (*Figure 1*), as discussed.

Noncovalent bonds were disrupted with the addition of SDS into the sample buffer, which generally increased the relative abundance of soluble aggregates, decreased that of hexameric and trimeric functional proteins, and slightly increased that of lower MW polypeptides among the samples (*Table 1, Figure 3B*). The addition of BME would cleave disulfide linkages, potentially increasing the relative abundance of soluble aggregates, legumin, and low MW polypeptides, while simultaneously decreasing the abundance of legumin monomers due to their reduction into acidic and basic subunits. Indeed, both cPPI and nPPI exhibited a significantly ($P < 0.05$) higher relative abundance

of soluble aggregates when solubilized in the presence of BME (*Table 1, Figure 3C compared to 3A*). However, the relative abundance of legumin and low MW polypeptides in cPPI and nPPI was hardly affected by the addition of BME. The partial dissociation of insoluble aggregates and soluble aggregates in the presence of BME masked the potential reduction of monomeric legumin into its low MW subunits, resulting in no observed change in the percent relative abundance. On the other hand, a significantly ($P < 0.05$) higher relative abundance of low MW polypeptides was observed across the isolated fractions, reconstituted isolates, and legumin-enriched nPPIs (*Figure 3C compared to 3A*). However, the percent relative abundance of soluble aggregates and functional proteins was hardly affected in these samples (*Table 1*). This observation could be partially attributed to low abundance of insoluble aggregates in these samples compared to cPPI and nPPI. In addition, reduction of disulfide linkages could have been incomplete due to inaccessible disulfide bonds buried within large, polymerized proteins, especially in 100L and 20V-80L. Both of these samples had negligible changes in soluble aggregates under SDS and BME relative to the phosphate buffer alone (*Table 1*). The presence of these compact, polymerized proteins may limit protein functionality.

**Table 1.** Molecular weight and relative abundance of soluble aggregates, legumin, convicilin, and vicilin present in cPPI, nPPI, isolated fractions, reconstituted protein isolates, and legumin-enriched nPPIs with varying 7S vicilin to 11S legumin ratios as analyzed by size-exclusion high-performance liquid chromatography (SE-HPLC).

| Sample | Relative Abundance (%) of Protein Fractions[1] | | | | | | | | | | | |
|---|---|---|---|---|---|---|---|---|---|---|---|---|
| | Phosphate Buffer[2] | | | | Phosphate Buffer (0.1% SDS)[3] | | | | Phosphate Buffer (0.1% SDS+ 2.5% BME)[4] | | | |
| | Soluble aggregates ( > 450 kDa) | Legumin (~450 kDa) | Conviclin (~250 kDa) | Vicilin (~160 kDa) | Soluble aggregates | Legumin | Conviclin | Vicilin | Soluble aggregates | Legumin | Conviclin | Vicilin |
| cPPI | 21.7$^{a5}$ | 2.20$^{h}$ | 6.15$^{b}$ | 4.93$^{f}$ | 20.5$^{c}$ | 4.89$^{h}$ | 6.23$^{c}$ | 4.84$^{g}$ | 33.0$^{a}$ | 2.46$^{f}$ | 6.69$^{bc}$ | 3.46$^{ef}$ |
| nPPI | 4.55$^{b}$ | 20.7$^{e}$ | 8.49$^{a}$ | 11.8$^{d}$ | 11.4$^{e}$ | 16.9$^{f}$ | 8.48$^{a}$ | 12.4$^{d}$ | 17.5$^{e}$ | 17.4$^{d}$ | 7.72$^{ab}$ | 11.1$^{cd}$ |
| 100V | 2.01$^{b}$ | 5.08$^{g}$ | 4.44$^{d}$ | 48.3$^{a}$ | 2.40$^{g}$ | 3.02$^{i}$ | 5.32$^{d}$ | 47.5$^{a}$ | 2.20$^{g}$ | 3.06$^{f}$ | 8.76$^{a}$ | 36.4$^{a}$ |
| 80V-20L | 6.76$^{b}$ | 17.7$^{f}$ | 7.81$^{b}$ | 30.9$^{b}$ | 9.06$^{f}$ | 10.7$^{g}$ | 7.35$^{b}$ | 32.1$^{b}$ | 8.45$^{f}$ | 12.7$^{e}$ | 7.98$^{ab}$ | 27.4$^{b}$ |
| 50V-50L | 17.5$^{a}$ | 28.9$^{d}$ | 5.65$^{bc}$ | 16.5$^{c}$ | 21.2$^{c}$ | 26.1$^{d}$ | 5.77$^{cd}$ | 7.81$^{f}$ | 21.6$^{cd}$ | 25.3$^{b}$ | 5.08$^{cd}$ | 7.27$^{de}$ |
| nPPI-50LE | 6.48$^{b}$ | 31.4$^{c}$ | 6.06$^{b}$ | 7.36$^{e}$ | 17.4$^{d}$ | 20.4$^{e}$ | 3.62$^{fg}$ | 18.5$^{c}$ | 15.7$^{e}$ | 22.4$^{c}$ | 6.37$^{bc}$ | 15.0$^{c}$ |
| 20V-80L | 21.3$^{a}$ | 36.9$^{b}$ | 5.33$^{bcd}$ | 8.17$^{e}$ | 26.3$^{a}$ | 30.3$^{b}$ | 4.52$^{e}$ | 5.29$^{g}$ | 21.2$^{d}$ | 30.2$^{a}$ | 4.44$^{d}$ | 7.86$^{de}$ |
| nPPI-80LE | 17.6$^{a}$ | 37.9$^{b}$ | 4.51$^{cd}$ | 4.56$^{f}$ | 24.2$^{b}$ | 28.7$^{c}$ | 4.33$^{ef}$ | 9.00$^{e}$ | 25.1$^{b}$ | 29.9$^{a}$ | 3.51$^{de}$ | 4.40$^{ef}$ |
| 100L | 22.2$^{a}$ | 42.4$^{a}$ | 4.48$^{d}$ | 1.28$^{g}$ | 28.3$^{a}$ | 33.0$^{a}$ | 3.15$^{g}$ | 2.00$^{h}$ | 23.7$^{bc}$ | 30.9$^{a}$ | 2.63$^{e}$ | 2.30$^{f}$ |

[1]Relative abundance (%) is the area of a specific peak divided by the total peak area for that sample; [2]Samples were dissolved in pH 7 phosphate buffer; [3]Samples were dissolved in pH 7 phosphate buffer with the presence of 0.1% SDS; [4]Samples were dissolved in pH 7 phosphate buffer with the presence of 0.1% SDS and 2.5% BME; [5]Lowercase letters indicate significant differences among the means (n = 2) in each column, according to the Tukey-Kramer multiple means comparison test ($P < 0.05$).

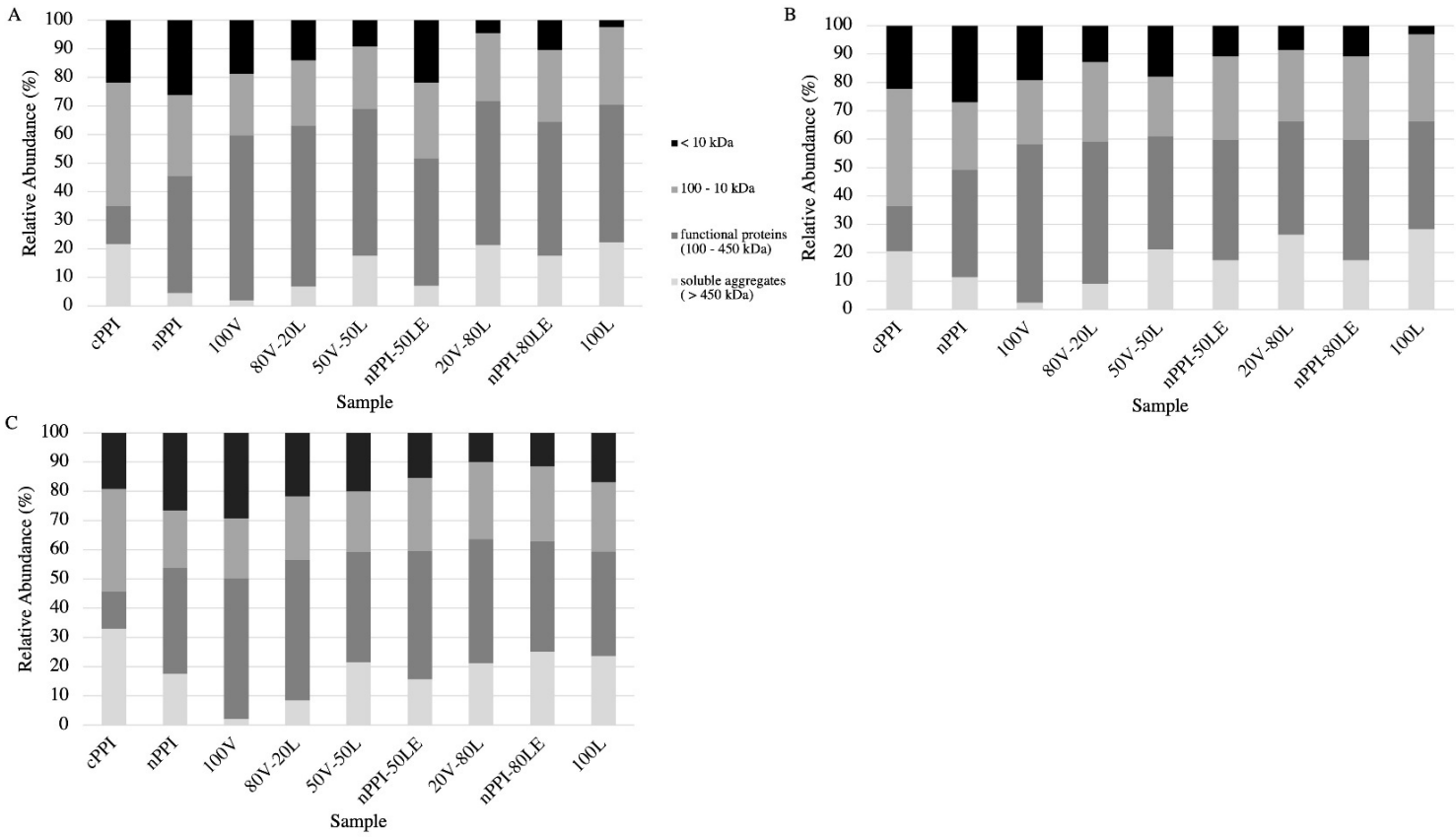


**Figure 3.** Percent relative abundance of different protein fractions in cPPI, nPPI, isolated fractions, reconstituted protein isolates, and legumin-enriched nPPIs. Samples were dissolved in (A) pH 7 phosphate buffer, (B) pH 7 phosphate buffer with 0.1% SDS, and (C) pH 7 phosphate buffer with 0.1% SDS and 2.5% BME, and analyzed by SE-HPLC. Bars distribution represents means of n = 2.

### 3.2.2 Protein denaturation state

Discrepancies in the reported thermal stability of pea protein have been partially attributed to intrinsic determinants such as protein profile and environmental conditions (Mession et al., 2015). Therefore, in this study, the impact of 7S/11S ratio and salt on protein denaturation temperature and enthalpy were evaluated.

Apart from cSPI and cPPI, which were already denatured and thus lacked a thermal transition (Bu et al., 2022, 2023), the remaining samples had either one prominent or two overlapping endothermic peaks. In the latter case, the two peaks were integrated as one due to the intersecting transition temperatures for 7S and 11S globulins (Mession et al., 2012). While the denaturation temperatures and enthalpies of the reconstituted isolates and legumin-enriched nPPIs were similar to previous reports on pea protein (Bu et al., 2022; Hansen et al., 2022; Kornet et al., 2021), those of 100V-salt and 100L-salt were significantly higher (*Table 2*). The latter observation was attributed to the stabilizing effect of NaCl on protein structure (Sun & Arntfield, 2010, 2012).

Among nPPI, nPPI-50LE, and nPPI-80LE, denaturation temperature and enthalpy increased with the relative abundance of 11S legumin. Legumin's thermal stability is attributed to its complex quaternary structure and intermolecular disulfide bridges, which require relatively high energy to disrupt (Mession et al., 2015). Further, when added to nPPI in high concentrations, the partially denatured isolated legumin fraction potentially induced a hydrophobicity driven protein association (*Figure 1*), stabilized by both hydrophobic and disulfide interactions (Rickert, Johnson, & Murphy 2004). Thus, nPPI-80LE's enthalpy of denaturation was significantly higher than that of nPPI-50LE and nPPI (*Table 2)*.

**Table 2.** Denaturation temperatures and enthalpy, surface hydrophobicity, and surface charge of cSPI, cPPI, nPPI, isolated fractions, reconstituted protein isolates, and legumin-enriched nPPIs with varying 7S vicilin to 11S legumin ratios.

| Sample | Denaturation Temperature and Enthalpy | | Surface Properties | |
|---|---|---|---|---|
| | Denaturation Temperature | Enthalpy of Denaturation | Surface Hydrophobicity | Surface Charge |
| | Td, °C | ΔH, J g-1 | RFI | mV |
| cSPI | *[1] | * | 11736[a2] | -41.9[a] |
| cPPI | * | * | 10261[b] | -34.8[bcde] |
| nPPI | 84.00[ef] | 7.10[c] | 8021[c] | -34.9[bcde] |
| 100V | 83.63[f] | 5.32[cd] | 4097[f] | -33.7[cde] |
| 100V-salt | 88.37[b] | 14.90[a] | 7177[cd] | -6.94[g] |
| 80V-20L | 84.88[def] | 3.44[d] | 5063[f] | -33.5[de] |
| 50V-50L | 85.38[cde] | 6.30[c] | 4840[f] | -33.4[e] |
| nPPI-50LE | 85.87[cd] | 7.31[c] | 7373[cd] | -36.6[b] |
| 20V-80L | 85.88[cd] | 3.15[d] | 5685[ef] | -35.9[bcde] |
| nPPI-80LE | 86.74[c] | 9.83[b] | 6617[de] | -36.4[bc] |
| 100L | 86.33[c] | 6.14[c] | 6027[e] | -36.1[bcd] |
| 100L-salt | 98.01[a] | 15.75[a] | 7610[c] | -10.2[f] |

[1]An asterisk (*) denotes no peak of denaturation observed; [2]Lowercase letters indicate significant differences among the means (n = 3) in each column, according to the Tukey-Kramer multiple means comparison test ($P < 0.05$).

Meanwhile, the isolated fractions and their corresponding reconstituted isolates had lower enthalpies of denaturation than nPPI and the legumin-enriched nPPIs. This finding was attributed to the partial denaturation that occurred during the extraction, as discussed. These differences in denaturation state among the samples will likely contribute to differences in other structural and in functional properties.

### 3.2.3 Protein surface properties

Although nonpolar residues typically inhabit ~40-50% of the water-accessible surface for most globular plant proteins, the ratio and distribution of polar to nonpolar regions are partially dictated by protein profile as well as amino acid composition and sequence (Damodaran & Parkin, 2017). The unique sequence of amino acids lining the protein's surface partially determines its overall surface hydrophobicity and charge, which will in turn affect functional behavior. Therefore, the impact of 7S/11S ratio and salt on surface properties were evaluated for all of the pea protein samples in reference to cSPI.

cSPI and cPPI had significantly the highest surface hydrophobicity, which complimented the observed denaturation data (*Table 2*), similar to previous reports (Bu et al., 2022; Hansen et al., 2022). Intramolecular electrostatic interactions, hydrogen bonding, and hydrophobic interactions that stabilize the protein are disrupted upon denaturation, leading to protein unfolding and exposure of the mostly hydrophobic core. Upon partial exposure of the hydrophobic core, the surface hydrophobicity is proportionally increased (Foegeding and Davis, 2011).

Among the fractionated and reconstituted isolates, 100L had the highest surface hydrophobicity, while 100V had the lowest (*Table 2*). This finding agreed with that of Mwasaru, Muhammad, Bakar, & Che Man (1999) and Barac et al. (2010), who reported legumin had more surface hydrophobic groups than vicilin. As previously discussed, the elaborate extraction steps and changes in their associated environmental conditions induced protein denaturation in 100L, which led to enhanced surface hydrophobicity relative to 100V. In contrast, the surface hydrophobicity of nPPI and the legumin-enriched nPPIs decreased as 11S legumin abundance increased. As discussed, the isolated legumin fraction induced a hydrophobicity driven protein polymerization when added to nPPI, which subsequently reduced the surface hydrophobicity of nPPI-50LE and nPPI-80LE (Wang & Ismail, 2012). This observation emphasized the compounded effect of denaturation and 7S/11S ratio on extent of polymerization and resultant surface properties. Meanwhile, the addition of NaCl in 100V-salt and 100L-salt led to significantly higher surface hydrophobicity compared to their 100V and 100L counterparts. NaCl likely shielded some charges on the surface of the protein, leading to higher perceived hydrophobicity (Damodaran, 1988).

Zeta potential (ζ) was measured as an indication of surface charge. All samples, except 100L-salt and 100V-salt, carried a highly net negative charge at pH 7 (*Table 2*), which was attributed to the charge shielding effect of NaCl (Damodaran, 1988). Samples with a higher 11S legumin abundance (100L, 20V-80L, nPPI-50LE, and nPPI-80LE) had higher net negative charge than 100V, with a few minor statistical differences. Danielsson (1949) determined the isoelectric points of legumin and vicilin as pH 4.8 and 5.5, respectively. Accordingly, at pH 7, legumin is further from its isoelectric point and

therefore carries a greater net negative charge than vicilin, which may explain the observed differences. The interplay of surface charge and surface hydrophobicity affect how the protein interacts with its environment, and therefore will impact functional behavior.

### 3.2.4 Protein secondary structures

The secondary structure profile of pea protein samples was deduced via the deconvolution of the Amide I peak. The secondary structure of cPPI was dominated by intermolecular β sheet (*Figure 4A*), indicating protein polymerization through hydrogen bonds. Additionally, random coil was prominent in cPPI. Meanwhile, the intermolecular β sheet and random coil were absent in nPPI; instead, β sheet, α helix, and β turn largely dominated the Amide I region (*Figure 4B*). This finding suggested that, in contrast to cPPI, the secondary structure of nPPI was mostly preserved due to mild extraction conditions.

In comparing the isolated fractions, β sheet and β turn dominated the Amide I region of 100V, while random coil, β sheet, α helix, and intermolecular β sheet were all prominant in that of 100L (*Figure 4, C & J*). This major difference in secondary structure between 100V and 100L was largely attributed to the intrinsic structural differences between vicilin and legumin (Barac et al., 2010). Further, random coil and intermolecular β sheet in 100L could be due to the fractionation process, in agreement with the SDS-PAGE observations (*Figure 1*), as discussed. However, the addition of salt decreased the relative abundance of the intermolecular β sheet and random coil in 100L and increased α helix in 100V (*Figure 4, D & K*). This observation indicated that the additional salt

stabilized the secondary structure of vicilin and legumin, which agreed with the enhanced denaturation enthalpy of 100V-salt and 100L-salt (*Table 2*).

Although α helix was not detected in 100V, the reconstituted protein isolates and legumin-enriched nPPIs had an apparent α helix region, attributed to the presence of legumin (*Figure 4, E, F, G, H, & I*). In addition, high abundance of legumin in reconstituted protein isolates contributed to the presence of intermolecular β sheet and random coil (*Figure 4, F & H*). Legumin-enriched nPPIs (*Figure 4, G & I*), on the other hand, were not largely impacted by the additional legumin, which could be attributed to the nPPI matrix effect. Protein secondary structure predominated by β sheet could be beneficial to some functional properties such as gelation and emulsification, whereas random coil and intermolecular β sheet could reduce protein solubility.

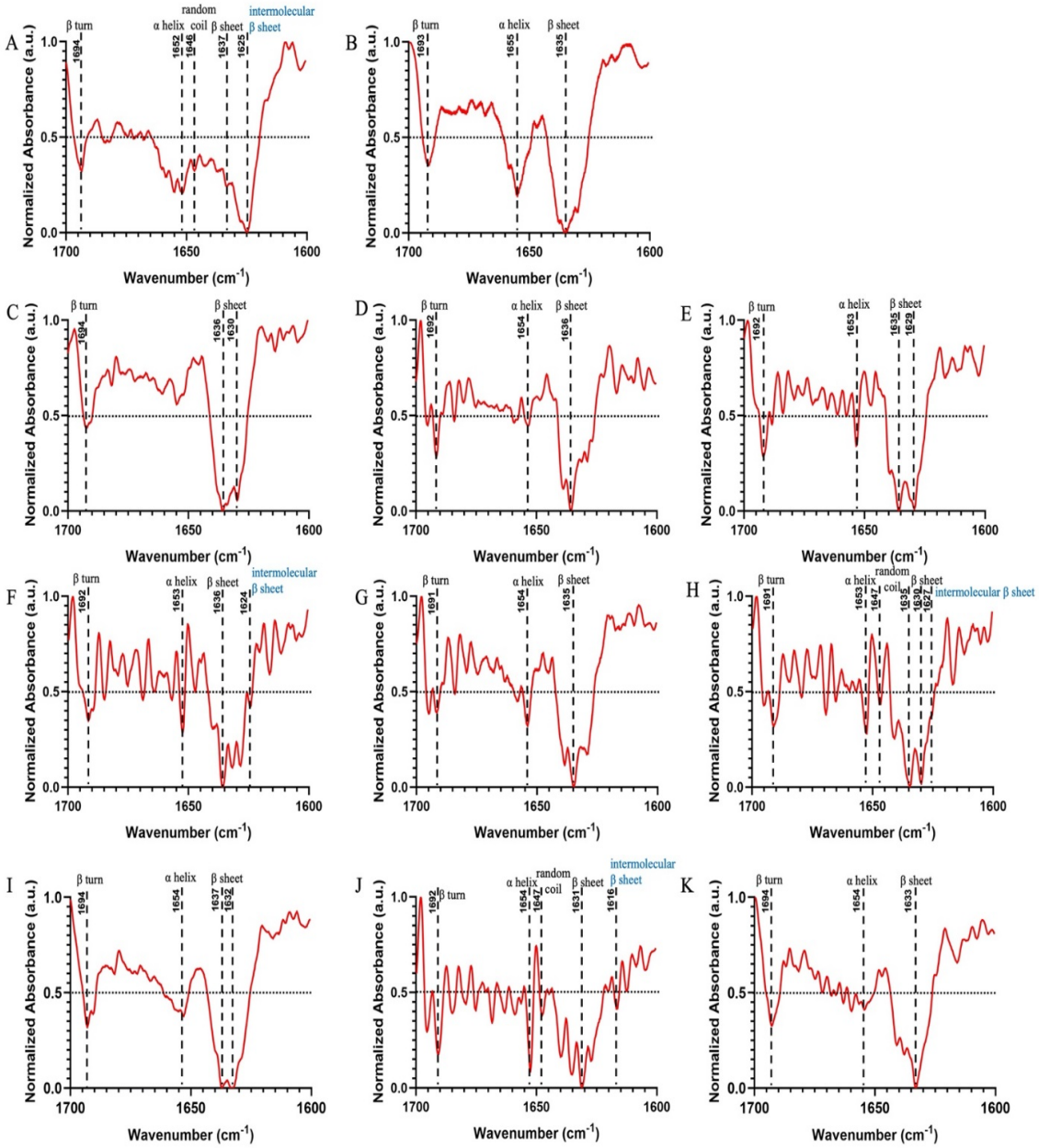


**Figure 4.** The second derivative of FTIR-ATR spectra. (A) cPPI (B) nPPI (C) 100V (D) 100V-salt (E) 80V-20L (F) 50V-50L (G) nPPI-50LE (H) 20V-80L (I) nPPI-80LE (J) 100L (K) 100L-salt

## 3.3 Functional properties of the isolated fractions, reconstituted protein isolates, and legumin-enriched nPPIs

### 3.3.1 Protein solubility

Protein solubility is a critical functionality for high protein beverages as well as food applications that involve other functional properties such as thickening, foaming, emulsifying, and gelling (Damodaran & Parkin, 2017). Protein solubility is governed by intrinsic characteristics such as molecular weight and surface properties and is also influenced by environmental conditions such as pH, ionic strength, and temperature (Damodaran & Parkin, 2017; Guo, Hu, Wang, & Ai, 2017). Therefore, in this study, the impact of 7S/11S ratios and salt on protein solubility were evaluated at neutral and acidic pH and under non-heated and heated conditions.

cPPI was significantly the least soluble among the samples across all conditions (*Table 3*), which was partially attributed to its denatured state (*Table 2*) and extent of polymerization (*Figure 1*). As discussed, denaturation often increases surface hydrophobicity, which will hinder protein-water interactions and decrease protein solubility. In contrast, despite its denatured state, cSPI was significantly more soluble than cPPI under neutral conditions. This finding is attributed to cSPI's significantly higher surface charge at pH 7 compared to cPPI (*Table 2*), which permitted sufficient protein-water interactions under neutral conditions. On the other hand, nPPI was significantly more soluble than the commercial ingredients across most conditions as it was less denatured and had lower surface hydrophobicity (*Table 2*).

In comparing the isolated fractions, 100V was significantly more soluble than 100L across all conditions (*Table 3*). This finding was partially attributed to 100V's

relatively less polymerization (*Figure 1*), significantly lower surface hydrophobicity, (*Table 2*), and lack of intermolecular β sheet and random coil compared to 100L (*Figure 4*). However, the intrinsic properties of pea vicilin, namely its lower molecular weight, glycosylated subunits, and high flexibility, likely contributed to 100V's high solubility across all conditions (Kimura et al., 2008; Maruyama et al., 2002; Pedrosa, Trisciuzzi, & Ferreira, 1997). Further, at pH 3.4, vicilin holds a greater net positive charge than legumin due to its higher isoelectric point (5.5 vs. 4.8), which may have further enhanced its interaction with water (Danielsson, 1949).

The isolated fractions, reconstituted isolates, and legumin-enriched nPPI samples had comparable or better solubility than nPPI across all conditions, except for 80V-20L at pH 7 (*Table 3*). This exception cannot be explained by differences in the surface properties of 80V-20L compared to the other samples (*Table 2*), but instead could be related to possible differences in ionic strength and other unclear compounded factors. At pH 3.4 specifically, the significantly lower solubility of nPPI compared to the produced pea protein samples could be partly attributed to its significantly higher surface hydrophobicity (*Table 2*) and ash content (~4% vs. 2%, data not shown). The low net charge of protein at this acidic pH, coupled with high ionic strength, reduce protein solubility (Kimura et. al, 2008). Therefore, at pH 3.4, the amount of salt in nPPI most likely had a charge-shielding effect on the protein and potentially competed for water, hindering protein-water interactions. This phenomenon was further demonstrated in 100V-salt and 100L-salt, as their solubility at pH 3.4 was significantly reduced compared to 100V and 100L counterparts. Further, heating 100V-salt and 100L-salt at 80ºC for 30 min drastically reduced solubility due to protein denaturation and a salting-out effect

(Damadoran, 1988). The increase in thermal kinetic energy under heated conditions likely enhanced hydrophobicity-driven protein-protein interactions and exacerbated the decrease in solubility (Damodaran & Parkin, 2017).

Meanwhile, the solubility of 100V-salt was not significantly different than that of 100V under neutral conditions (*Table 3*), despite the former's significantly higher surface hydrophobicity and lower surface charge (*Table 2*). Vicilin's intrinsic structure, which favors solubility, potentially overcame the environmental effects imposed by salt to remain highly soluble under neutral pH. A similar phenomenon has been demonstrated with β-lactoglobulin, as its unique structure allows high solubility under harsh environmental conditions (Damodaran & Parkin, 2017). On the other hand, 100L-salt was significantly more soluble than 100L under neutral conditions. This finding can be related to the protein secondary structure observations, where 100L-salt had lower relative abundance of intermolecular β sheet and random coil than 100L (*Figure 4)*. Further, at certain concentrations and at specific pH, salt can uniquely affect electrostatic interactions and the bulk water structure around the protein, which likely enhanced hydration and increased protein solubility for 100L-salt (Hill, Ledward, & Mitchell, 1998; Meng & Ma, 2001; Von Hippel & Schleich, 1969).

While 100V was more soluble than 100L across all conditions, samples with higher 7S/11S ratio did not follow suit. Compounded environmental factors and differences in the protein structure, as influenced by fractionation conditions, contributed to differences in solubility more than differences in 7S/11S ratio did. Isolated environmental conditions need to be further explored to differentiate the impact of each factor from the 7S/11S ratio on protein solubility.

**Table 3.** Solubility, gel strength and emulsification capacity of cSPI, cPPI, nPPI, isolated fractions, reconstituted protein isolates, and legumin-enriched nPPIs with varying 7S vicilin to 11S legumin ratios.

| Sample | Solubility (5% protein) | | | | Gel Strength (20% protein) | Emulsification Capacity (2% protein) |
|---|---|---|---|---|---|---|
| | pH 7 | | pH 3.4 | | | |
| | Non-Heated | Heated (80°C for 30 min) | Non-Heated | Heated (80°C for 30 min) | Strength (N) | mL oil/g protein |
| cSPI | 73.5[b1] | 86.1[ab] | 28.1[f] | 38.3[f] | 16.69[a2] | 1102.1[a3] |
| cPPI | 31.4[d] | 54.2[e] | 12.5[g] | 18.0[g] | 8.37[d] | 725.4[b] |
| nPPI | 85.5[a] | 81.4[bc] | 49.8[e] | 62.4[e] | 4.62[f] | 502.2[efg] |
| 100V | 91.4[a] | 74.9[cd] | 95.4[a] | 96.0[a] | 13.8[b] | 697.5[bc] |
| 100V-salt | 93.3[a] | 73.9[cd] | 75.8[cd] | 6.95[h] | 17.2[a] | 534.8[ef] |
| 80V-20L | 63.7[c] | 61.8[e] | 92.3[a] | 90.3[ab] | 11.5[c] | 589.0[cde] |
| 50V-50L | 89.8[a] | 72.7[d] | 83.9[b] | 87.6[bc] | 6.31[e] | 568.9[de] |
| nPPI-50LE | 88.6[a] | 81.2[bc] | 72.9[d] | 78.0[d] | *[4] | 434.0[fg] |
| 20V-80L | 86.0[a] | 72.8[d] | 83.2[b] | 88.1[bc] | 2.66[g] | 545.6[ef] |
| nPPI-80LE | 92.4[a] | 89.6[a] | 78.0[c] | 83.4[cd] | 1.31[h] | 393.7[g] |
| 100L | 69.7[bc] | 62.0[e] | 79.5[bc] | 81.4[cd] | 2.61[g] | 130.2[h] |
| 100L-salt | 87.2[a] | 85.7[ab] | 49.9[e] | 18.1[g] | 2.26[gh] | 663.4[bcd] |

[1]Lowercase letters indicate significant differences among the means (n = 3) in each column, according to the Tukey-Kramer multiple means comparison test ($P < 0.05$); [2]Gel strength was measured at 15% protein concentration for cSPI; [3]Emulsification capacity was measured at 1% protein concentration for cSPI; [4]An asterisk (*) denotes no gel formed at 20% protein concentration.

### 3.3.2 Gel strength

Like solubility, gelation properties are greatly impacted by the inherent protein profile. In soy protein, cysteine residues in 11S glycinin are especially important in forming inter- and intramolecular disulfide linkages, which are critical to establishing and strengthening the gel network (Hermansson, 1986; Nakamura, Utsumi, Kitamura, Harada, & Mori, 1984). However, for pea protein, hydrophobic interactions, hydrogen bonds, and electrostatic interactions have greater contribution to gel formation and strength than disulfide linkages (O'Kane, Happe, Vereijken, Gruppen, & Van Boekel, 2004b; Sun & Arntfield, 2010, 2012). The lower contribution of disulfide linkages to gel formation in pea protein compared to soy protein could be attributed to inherent differences in the structure and composition of 11S between species. Given these differences, it is unclear how 7S/11S ratio in pea could affect the reactions critical to gel formation. Therefore, in this study, the impact of 7S/11S ratios and salt on pea protein gel strength were evaluated.

cSPI formed a significantly stronger gel at 15% protein concentration than any of the pea protein samples at 20% protein concentration (*Table 3*), attributed to soy protein's inherent molecular properties. This observation was similar to previous reports comparing soy protein to pea protein gels (Hansen et al., 2022; O'Kane et al., 2004b; Shand,Ya, Pietrasik, & Wanasundara, 2007; Sun & Artnfield, 2012). Meanwhile, nPPI formed a significantly weaker gel structure than cPPI, attributed to its relatively native protein state and significantly lower surface hydrophobicity (*Table 2*), which potentially reduced the protein-protein interactions necessary for gel network formation.

Among the isolated fractions and reconstituted isolates, gel strength increased with the abundance of vicilin (*Table 3*). 100V had low extent of polymerization (*Figure 1*), was partially denatured (*Table 2*), and had high relative abundance of β sheet (*Figure 4*), allowing it to form an organized gel network upon heating. Further, 100V's relatively low surface hydrophobicity and net surface charge might have achieved an ideal balance between attractive and repulsive forces, which contributed to the formation of a relatively strong gel network. In contrast, multiple factors inhibited strong gel formation for 100L. First, 100L was highly polymerized by disulfide linkages (*Figure 1)* and intermolecular β sheet interactions (*Figure 4*), which potentially caused the formation of insoluble aggregates, rather than a uniform gel network, upon heating. Large polymers also restrict strand flexibility during heating (O'Kane et al., 2005), which could have ultimately weakened the protein-protein interactions critical to gel formation for 100L. Secondly, the denaturation temperature of 100L was significantly higher than that of 100V (*Table 2*); therefore, 100L had greater resistance to unfolding than 100V during thermal treatment. Consequently, reactive sulfhydryl groups mostly likely remained buried within 100L's core and were thus inaccessible for gel formation. Additionally, the abundance of disulfide-linked polymers (*Figure 1*) in 100L potentially reduced the availability of sulfhydryl groups needed for gel formation. Results also showed that with increasing abundance of 11S legumin in the reconstituted isolates and legumin-enriched nPPIs, there was a significant decrease in gel strength (*Table 3*). The discussed physicochemical properties of legumin also potentially interfered with the hydrophobic interactions, hydrogen bonding, and electrostatic interactions needed to form a strong gel.

Meanwhile, the effect of salt addition was significant for 100V but not for 100L. 100V-salt had a significantly higher gel strength than 100V (*Table 3*). The salt had a charge shielding effect, evidenced by the perceived higher surface hydrophobicity and lower surface charge (*Table 2*), which enhanced the noncovalent interactions among the 7S vicilin protein molecules and increased gel strength. On the other hand, the addition of salt in 100L enhanced the rigidity of the legumin proteins, evidenced by the significantly higher denaturation temperature and enthalpy (*Table 2*). This stabilized protein structure in 100L-salt prevented the legumin proteins from unfolding, which is a necessary prerequisite for gel formation.

Considering the homology between pea and soy protein, the inferior gelling properties of the former has been frequently attributed to the lower abundance of 11S in pea compared to soy. However, results of this work contradicted this assumption. Intrinsic differences between the two protein sources, in addition to the considerable variability in pea 11S legumin performance across different studies, must be alternatively considered. Similar to the results of this study, Barac et al. (2010), Bora et al. (1994), and Mession et al., (2015) reported that legumin formed weak gels. In contrast, O'Kane et al. (2004a, 2004b, 2005) produced strong gels from pea legumin fractions at protein concentrations as low as 10.5%. This controversy in the reported gelation properties of pea legumin is in stark contrast to the consistent gelling performance of soy 11S glycinin across different studies. The consistent gelling performance of soy 11S glycinin is attributed to years of breeding efforts to eliminate molecular variability (Mertens et al., 2012; Murphy & Resurreccion, 1984). In particular, the gelling performance of soy protein is attributed to soy glycinin consistently containing 8 cysteine residues per

subunit, while pea legumin may contain anywhere from 2-7 (Casey & Short; 1981; Croy, Gatehouse, Tyler, & Boulter, 1980; O'Kane et al., 2004b; Mession, Sok, Assifaoui, & Saurel, 2013). The relative lack of research on the inherent molecular properties of pea legumin, coupled with its wide compositional diversity both within and among cultivars, necessitate additional studies relating pea 11S legumin to gelation as well as other functional properties.

### *3.3.3 Emulsification capacity*

cSPI had significantly higher EC than all pea protein samples (*Table 3*). Compared to the pea protein samples, cSPI had a favorable balance between surface hydrophobicity and surface charge (*Table 2*), which potentially allowed for efficient migration to the interface and the formation of protein films around the oil droplets. In contrast, cPPI had significantly lower surface charge and solubility than cSPI (*Table 2 & 3*), which potentially caused a relatively unfavorable hydrophilic/lipophilic balance and significantly lower EC. Nevertheless, cPPI had a significantly higher EC than nPPI (*Table 3*). Compared to cPPI, nPPI was less denatured, had significantly lower surface hydrophobicity, and significantly higher solubility (*Table 2 & 3*), which potentially disrupted nPPI's hydrophilic/lipophilic balance in favor of protein-water interactions.

Between the isolated fractions, 100V had a significantly higher EC than 100L (*Table 3*). This difference could be attributed to certain intrinsic characteristics of vicilin including its low molecular weight, which facilitates fast migration to the interface. Further, the high flexibility of vicilin due to lack of disulfide linkages allows it to easily orient at the interface (Liang & Tang, 2013). In contrast, legumin's high molecular

weight and more compact structure may hinder migration to and orientation at the interface (Mession et al., 2015). Additionally, 100V was less polymerized (*Figure 1*), had a high relative abundance of β sheet (*Figure 4*), and had significantly higher solubility compared to 100L (*Table 3*), allowing it to have a relatively more balanced protein-water and protein-protein interactions at the interface.

Meanwhile, salt had a varied effect on the EC of the isolated fractions (*Table 3*). 100V-salt had a significantly lower EC than 100V, attributed to the salt's charge shielding effect (*Table 2*), which negatively impacted the hydrophilic/lipophilic balance. However, addition of salt to 100L potentially increased the charge load on the protein, leading to enhanced protein-water interactions and improved hydrophilic/lipophilic balance, which consequently increased EC.

In comparison to 100V, increasing the proportion of 11S legumin negatively impacted EC, with significant reductions observed for 50V-50L, 20V-80L, and nPPI-80L. However, enriching nPPI with the low-performing fractionated legumin did not significantly cause further detriment to the EC. On the other hand, both nPPI-50L and nPPI-80L had significantly lower EC than 50V-50L and 20V-80L, respectively. These observations suggested that structural differences between legumin and vicilin imposed by fractionation, as well as the matrix effect, contributed more to the differences in EC than varying the 7S/11S ratio. The high polymerization and low solubility of the fractionated legumin, in comparison to fractionated vicilin, contributed the most to the observed differences in EC. Therefore, the impact of varying 7S/11S ratio on the emulsification behavior was not clearly distinguished.

### 3.4 Amino acid composition and PDCAAS of isolated fractions, reconstituted isolates, and legumin-enriched nPPIs

The amino acid composition of pea protein samples was evaluated to determine the proportion of key amino acids for structural and functional consideration. In general, all samples were rich in acidic amino acids but had a low percentage of sulfur-containing amino acids (*Table 4*). This finding agreed with previous reports of the amino acid composition of pea protein (Rutherfurd et al., 2014). Significant differences in the percent distribution of amino acids, except for that of hydrophobic amino acids, were noted among the samples.

cPPI had a significantly lower abundance of acidic and basic amino acids relative to the other samples, with the exception of 100V for acidic amino acids. This finding may be attributed to degradation of amino acids under the harsh extraction parameters typically used to produce cPPI. For example, lysine is especially sensitive to alkaline pH, higher extraction temperature, and longer extraction time (Feyzi, Varidi, Zare, & Varidi, 2018). Additionally, both aspartic and glutamic acid are deamidated under alkaline conditions coupled with adverse heat treatment (Damodaran & Parkin, 2017). Amino acid degradation can influence functional properties and nutritional quality of protein ingredients.

100L had a significantly higher content of sulfur-containing amino acids than 100V (*Table 4*). For the most part, samples with higher proportion of 11S legumin had a significantly higher percentage of sulfur-containing amino acids relative to nPPI and 100V. This observation was consistent with previous reports, though the amino acid composition of 11S legumin varied considerably within and across cultivars (Casey &

Short, 1981; Choi, Taghvaei, Smith, & Ganjyal, 2022; Mession et al., 2013; Rangel, Domont, Pedrosa, & Ferreira, 2003). This reported variation could explain the lack of significant difference in the amount of sulfur-containing amino acids between cPPI and 50V-50L or 20V-80L, despite their high abundance of 11S legumin. However, the high relative abundance of sulfur-containing amino acids did not correspond to enhanced gelling properties (*Tables 3 & 4*). This observation might indicate that the cysteine residues in 11S legumin in pea have little contribution to gel strength, in stark contrast to soy 11S glycinin, as discussed. However, the noted polymerization of the isolated 11S legumin (*Figure 1, lane 10*), could have contributed to a comparatively low gel strength.

Significant differences between 100L and 100V were also noted in their percentage of acidic amino acids and basic amino acids (*Table 4*). 100L contained significantly more acidic and basic amino acids than 100V. The latter finding agreed with Barac et al. (2010), who reported 7S vicilin contained less basic amino acids than 11S legumin. Since 100V was lower in both acidic and basic amino acids compared to 100L, the ratio was not significantly different from the other samples. The relative ratio of acidic and basic amino acids determines the net charge on the surface of protein (Tang, Chen, & Ma, 2009). This observation is consistent with the lack of significant difference in zeta potential at pH 7 (*Table 2*).

On the other hand, 100V contained significantly more critical amino acids than 100L. There were a few minor statistical differences in the abundance of critical amino acids among the remaining pea protein samples. However, as the percentage of critical amino acids for all pea protein samples were below the theoretical critical value of 28%, this difference likely imparted negligible practical impact on functionality among the

samples (Mo, Zhong, Wang, & Sun, 2006). Of the hydrophobic amino acids, phenylalanine, leucine, isoleucine, and valine are critical because of their degree of hydrophobicity compared to other hydrophobic residues (Mo et al. 2006). If these amino acids comprise more than 28% of the total amino acids, hydrophobic interactions will offset any electrostatic interactions, resulting in protein aggregation and minimal solubility across a wide pH range. The percentages of these critical amino acids in all samples were similar to what is reported for the acidic subunit (20.1%) of soy 11S glycinin (Mo et al. 2006). This observation could partially explain the good overall solubility of nPPI, 100V, 100L, reconstituted and legumin enriched nPPI samples at both pH 7 and 3.4 (*Table 3*).

In terms of nutritional quality, all pea protein samples had high *in vitro* digestibility (≥ 100%, *Table 6, Appendix H*). Thus, their PDCAAS was equal to the amino acid score (AAS) (*Table 4*). The PDCAAS of 100L was significantly higher than that of 100V. Consequently, increasing the abundance of 11S legumin significantly raised the PDCAAS of reconstituted isolates compared to 100V. On the other hand, legumin enrichment did not improve the PDCAAS of either nPPI-50LE or nPPI-80LE (*Table 4*). While nPPI was limited by cysteine and methionine, similar to what was reported by Rutherfurd et al. (2014), cPPI, 100L, legumin-enriched nPPIs, 20V-80L, and 50V-50L were limited by threonine (data not shown). Cysteine, methionine, and threonine are among the most susceptible amino acids to alkaline pH, long extraction time, and higher extraction and drying temperatures (Damodaran & Parkin, 2017; De Groot & Slump, 1969; Feyzi et al., 2018). Thus, relative to nPPI, harsher extraction parameters for the isolated fractions and cPPI may have degraded susceptible amino acid residues, resulting

in reduced PDCAAS. Meanwhile, while threonine and sulfur-containing amino acids were also low in 100V and 80V-20L, tryptophan was the limiting amino acid in these samples. This observation was consistent with previous reports of vicilin containing significantly less tryptophan than legumin (Danielsson, 1949; Derbyshire et al., 1976; Rangel et al., 2003). Ultimately, the observed variation in PDCAAS among cPPI, nPPI, and the reconstituted and enriched samples may be in part due to the protein extraction conditions.

**Table 4.** Key amino acid (AA) percentage (g AA/100 g sample) and protein digestibility-corrected amino acid score (PDCAAS) of cPPI, nPPI, isolated fractions, reconstituted protein isolates, and legumin-enriched nPPIs with varying 7S vicilin to 11S legumin ratios.

| Sample | Sulfur-containing AA (%) | Acidic AA (%) | Basic AA (%) | Ratio of Acidic to Basic AA | Hydrophobic AA (%) | Critical AA (%) | PDCAAS[1] |
|---|---|---|---|---|---|---|---|
| cPPI | 2.25$^{cd2}$ | 28.3$^{c}$ | 10.3$^{d}$ | 2.74$^{a}$ | 25.7[3] | 13.4$^{b}$ | 0.630$^{d*}$ |
| nPPI | 1.81$^{ef}$ | 35.2$^{a}$ | 15.8$^{ab}$ | 2.23$^{b}$ | 27.9 | 15.7$^{ab}$ | 0.800$^{a}$ |
| 100V | 1.50$^{f}$ | 28.8$^{c}$ | 12.8$^{c}$ | 2.24$^{b}$ | 26.4 | 17.2$^{ab}$ | 0.389$^{e}$ |
| 80V-20L | 1.59$^{ef}$ | 34.0$^{ab}$ | 16.1$^{ab}$ | 2.11$^{b}$ | 29.9 | 19.0$^{a}$ | 0.594$^{d}$ |
| 50V-50L | 1.91$^{de}$ | 33.0$^{ab}$ | 16.3$^{a}$ | 2.02$^{b}$ | 30.6 | 18.9$^{a}$ | 0.649$^{cd}$ |
| nPPI-50LE | 3.03$^{b}$ | 30.6$^{bc}$ | 14.5$^{bc}$ | 2.12$^{b}$ | 27.1 | 14.8$^{ab}$ | 0.632$^{d}$ |
| 20V-80L | 2.49$^{c}$ | 33.2$^{ab}$ | 16.1$^{ab}$ | 2.07$^{b}$ | 29.8 | 17.8$^{a}$ | 0.654$^{bcd}$ |
| nPPI-80LE | 3.02$^{b}$ | 35.1$^{a}$ | 17.0$^{a}$ | 2.07$^{b}$ | 30.4 | 16.9$^{ab}$ | 0.736$^{abc}$ |
| 100L | 3.87$^{a}$ | 36.2$^{a}$ | 17.4$^{a}$ | 2.08$^{b}$ | 29.6 | 16.5$^{ab}$ | 0.742$^{ab}$ |

[1]Calculated based on the recommended amino acid scoring pattern for children (2 to 5 years) (FAO/WHO Expert Consultation, 1991) and an *in vitro* digestibility of ≥100% measured for all pea protein samples; [2]Lowercase letters indicate significant differences among the means (n = 3; n = 2 for PDCAAS) in each column, according to the Tukey-Kramer multiple means comparison test ($P < 0.05$); [3]No significant differences in hydrophobic amino acids were detected among pea protein samples, according to the Tukey-Kramer multiple means comparison test ($P < 0.05$)

# Chapter 4: Conclusions, Implications, and Recommendations

This study was the first to thoroughly characterize the impact of 7S/11S ratio on pea protein structure, functionality, and nutritional quality. Fractions of 7S vicilin and 11S legumin were successfully isolated from pea flour, and aliquots of each fraction were combined to produce samples of selected 7S/11S ratios. Additionally, for the first time, pea protein isolate was enriched with 11S legumin to evaluate the impact of higher 11S abundance on functionality and nutritional quality within an unfractionated protein matrix.

In soy protein, both the higher abundance of 11S glycinin and its sulfur-containing amino acids are credited for the superior functionality and nutritional quality of soy protein compared to pea protein. However, the results of this study were in stark contrast to what has been documented for soy protein. 11S legumin enrichment within the pea protein isolate matrix did not improve protein functionality or nutritional quality. In fact, the isolated 7S vicilin fraction had greater solubility, gel strength, and emulsification capacity than the isolated 11S legumin fraction. Therefore, the findings of this study shed doubt on the common assumption that the differences in 11S abundance between pea and soy protein is the main reason behind the former's inferior functionality. Rather, the results agreed with the hypothesis that there are certain physicochemical distinctions in pea 7S and 11S globulins, relative to their counterparts in soy protein, that contribute considerably to pea protein's inferior functional behavior and nutritional quality.

Another pertinent challenge tied to pea protein is its unpredictable functional behavior and nutritional quality. This variability, which goes beyond differences in

protein extraction and processing conditions, exacerbates the difficulty of utilizing pea protein ingredients to replicate soy protein functionality and nutritional quality consistently in the industry. This heterogeneity in pea protein can largely be attributed to genetic origin and agronomic factors and environmental conditions during the cultivation period. Thus, pea protein structure, functionality, and nutritional quality may vary widely within and across pea cultivars, from region to region, and from year after year. So, while the findings of this study indicated that 7S vicilin has greater functionality, but lower nutritional quality, than 11S legumin, further investigation is warranted to determine the isolated impact of genetic variance and molecular heterogeneity on pea protein functionality and nutritional quality.

Lastly, although fractionation was successful in producing enriched 7S and 11S fractions from pea flour, the process resulted in unique structural changes for each fraction. The proteins within the 11S fraction were more polymerized than those in the 7S fraction, which impacted their surface properties and secondary structure. These structural changes contributed to the observed functionality and nutritional quality of the 11S fraction, which convoluted the effect of the 7S/11S ratio. Nevertheless, this work provided foundational knowledge for future work aiming to devise strategies, such as targeted breeding programs, to predict and improve the quality and consistency of pea protein ingredients. Fostering and investing in such integrated efforts among interdisciplinary researchers and industry would have a critical impact on the continual development of pea as a source of functional and nutritious protein for the global food supply.

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# Appendix A: Sample calculation for preparation of legumin-enriched nPPIs

*Given:*

The protein profile of nPPI (86.9% protein purity) was determined by SDS-PAGE-densitometry to be ~20% vicilin, 80% legumin

*Then:*

To prepare nPPI-50LE (50% vicilin, 50% legumin) starting from 5 grams of nPPI:

$$5\ g\ nPPI * 0.869 * 0.8 = 3.476\ g\ vicilin$$

$$5\ g\ nPPI * 0.869 * 0.2 = 0.869\ g\ legumin$$

Protein purity of 100L is 100%, so

$$3.476 - 0.869 = 2.607$$

Add 2.607 g 100L to the 5 g nPPI to achieve ~50% legumin concentration

## Appendix B: Sample calculation for determining ionic strength of NaCl solution

$$I = \frac{1}{2}\sum(c_i \cdot z_i^2)$$

*where I = ionic strength; $c_i$ = concentration of ions, and $z_i^2$ = charges of ions squared*

*Given:*

When dissociated, NaCl produces one sodium ($Na^+$) and one chloride ($Cl^-$) ion, so the charges of ions are:

$$z_i(Na^+) = +1$$
$$z_i(Cl^-) = -1$$

*Then:*

Calculations for determining the concentration of ions in a 0.5M NaCl solution:

$$c_i(Na) = 0.5\ x\ 1 = 0.5\ mol/L$$
$$c_i(Cl) = 0.5\ x\ 1 = 0.5\ mol/L$$

Calculation for determining ionic strength of 0.5M NaCl solution:

$$I = \frac{1}{2}\sum\left(0.5\frac{mol}{L}\cdot 1^2\right)\left(0.5\frac{mol}{L}\cdot(-1)^2\right)$$
$$I = 0.5\ \frac{mol}{L}$$

# Appendix C: Sample calculation for ionic strength correction for fractions for functionality testing

*Given:*

Ash content of 100V: 2.19%. Assuming all of the salt in the samples is NaCl, 5 g of 100V contains:

$$5g * .0219 = 0.1095\ g\ NaCl$$

*And, as demonstrated in Appendix B:*

$$0.5\mu = 0.5\text{M NaCl} = \frac{29.22\ g\ NaCl}{1{,}000\ mL\ DDW}$$

Sample calculation to prepare 10% w/v protein solution (5 g in 50 mL) at 0.5 $\mu$ for functionality testing:

$$\frac{29.22\ g\ NaCl}{1{,}000\ mL\ DDW} x\ 50\ mL = 1.461\ g\ NaCl$$

$$1.461\ g\ NaCl - 0.1095\ g\ NaCl = \text{add } 1.3515\ g\ NaCl \text{ to the 10\% protein solution}$$

# Appendix D: Chromatograms and calibration curve for the standard proteins on SE-HPLC column

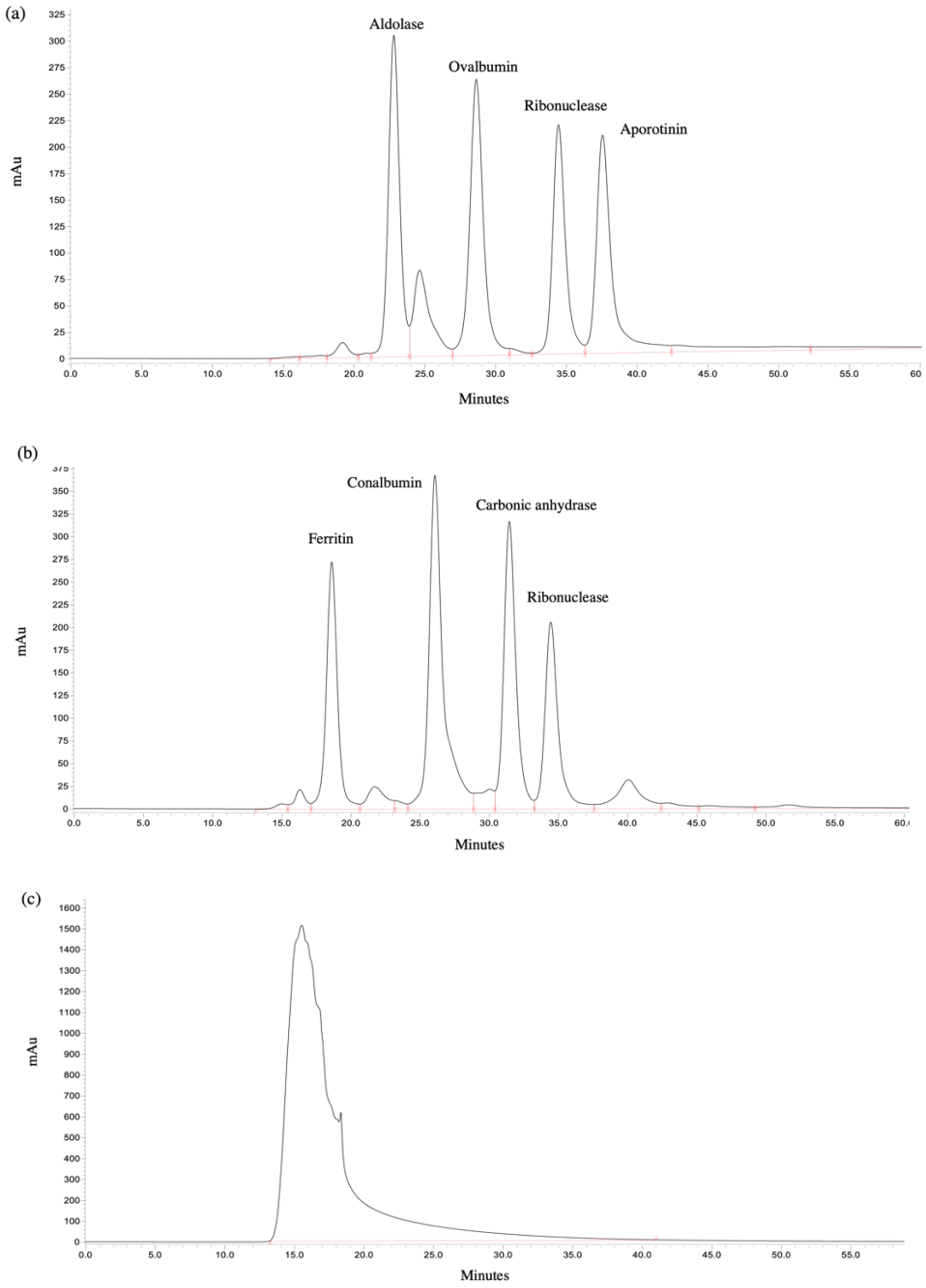


**Figure 5.** Chromatographic separation for the (a) low molecular weight and (b) high molecular weight standard proteins (c) blue dextrin on Superdex 200 Increase 10/300 GL column. Standard proteins, ferritin (440 kDa), aldolase (158 kDa), conalbumin (75 kDa), ovalbumin (43 kDa),

carbonic anhydrase (29 kDa), ribonuclease (13.7 kDa), and aprotinin (6.5 kDa), were used to calibrate the column.

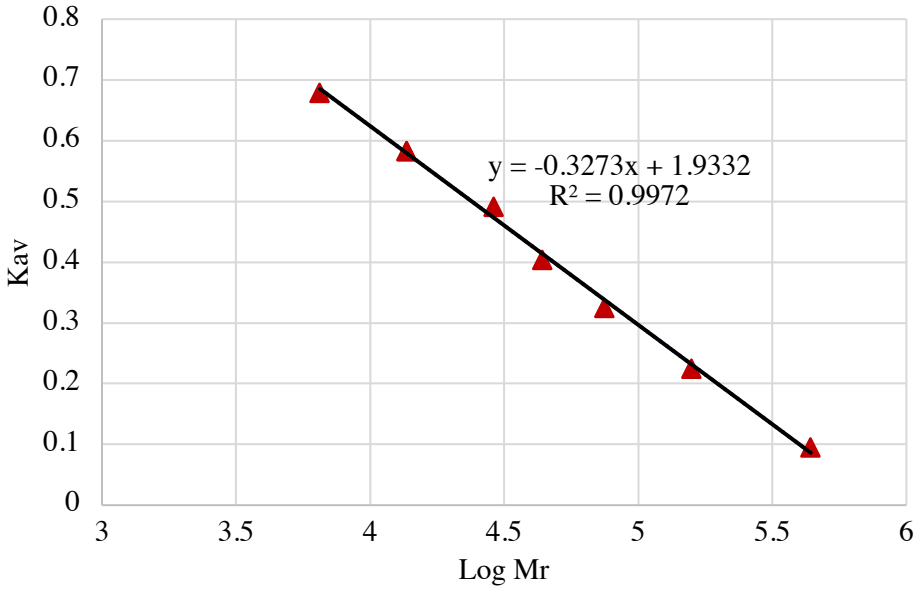


**Figure 6.** Calibration curve for the standard proteins on Superdex 200 Increase 10/300 GL column.

Sample calculation for protein size:

$$Kav = \frac{Ve-Vo}{Vc-Vo}$$

$$Vo = 7.77\ mL$$

$$Vc = 24mL$$

$$\text{Kav} = -0.3273\text{LogMr} + 1.9332$$

*Then:*

$$\text{Molecular weight (Da)} = 10^{\frac{\frac{Ve-7.77}{24-7.77}-1.9332}{0.3273}}$$

The elution volume of legumin is 9.334 mL, so the molecular weight of legumin is 407.9 kDa.

# Appendix E: Sample Calculation for Determining Surface Hydrophobicity Index

$$\text{Net Relative Fluorescence Intensity (RFI)} = RFI_{final} - RFI_{initial}$$

$$RFI_{final} = E_{sample.final} - E_{blank.final}$$

*Where:*

$E_{sample.final}$ = fluorescence emission of protein sample after ANS probe is added

$E_{blank.final}$ = fluorescence emission of buffer blank after ANS probe is added

$$RFI_{initial} = E_{sample.initial} - E_{blank.initial}$$

*Where:*

$E_{sample.initial}$ = fluorescence emission of protein sample before ANS probe is added

$E_{blank.initial}$ = fluorescence emission of buffer blank before ANS probe is added

*Given:* nPPI replicate at 0.050% protein (w/v)

$$RFI_{final} = 428 - 19.7 = 399.2$$

$$RFI_{initial} = 12.0 - 19.7 = -7.7$$

$$Net\ RFI = 399.2 + 7.7 = 406.8$$

Net RFI values for all protein solution concentrations (0.050%, 0.025%, 0.020%, 0.015%, 0.010%, and 0.005%) are plotted against protein concentration (*Figure 7*).

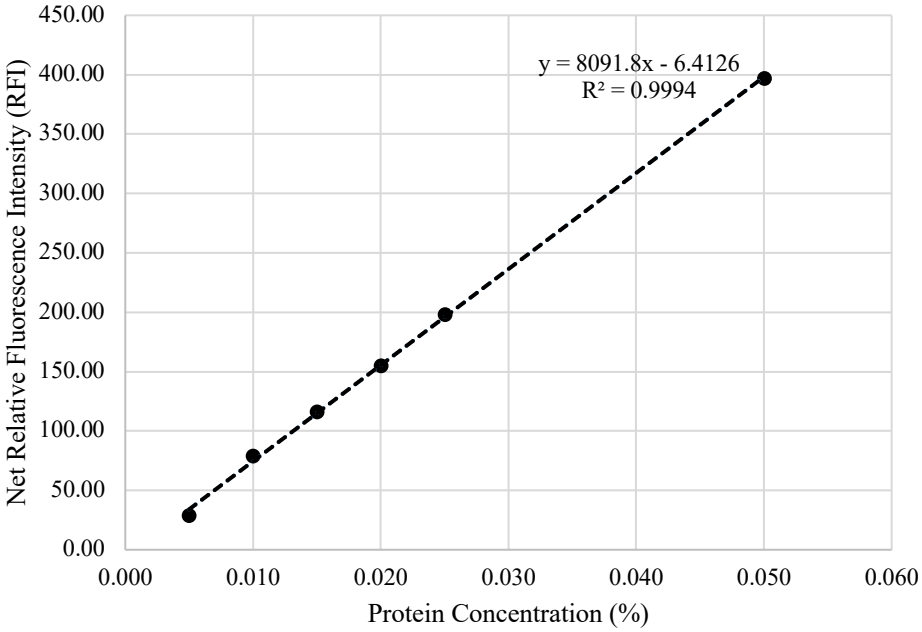


**Figure 7.** Net Relative Fluorescence Intensity (RFI) plotted against protein concentration (%) for nPPI replicate, to determine surface hydrophobicity index.

The slope of the linear trendline in *Figure 7* is the surface hydrophobicity index (8091.8). The final value for surface hydrophobicity index is the average of three replicates.

## Appendix F: Sample Calculation for Determining Protein Solubility

$$\%\ solubility = \frac{\%\ protein_{supernatant}}{\%\ protein_{initial}} * 100$$

*Given:*

One nPPI replicate (5% protein (w/v) at pH 3.4, non-heated) had 2.56% protein detected in the supernatant and 4.98% detected initially.

*Then:*

$$\%\ solubility = \frac{2.56\%}{4.98\%} * 100 = 51.41\%$$

## Appendix G: Sample Calculation for Determining Emulsification Capacity

$$\text{EC} = \frac{\text{volume of oil titrated (mL) x density of oil } (\frac{\text{g}}{\text{mL}})}{\text{mass of protein (g)}}$$

*Given:*

55 mL of oil was titrated for one replicate of nPPI

*And*

0.93 g/mL = density of corn oil

0.1 g = grams of protein in 5 mL of a 2% protein solution

$$\text{EC} = \frac{55 \text{ mL x } 0.93 \frac{\text{g}}{\text{mL}}}{0.1 \text{ g}} = 511.5 \ \frac{\text{g protein}}{\text{g oil}}$$

## Appendix H: Chromatogram for the amino acid standard and calibration curve for an amino acid on Waters UPLC-BEH column

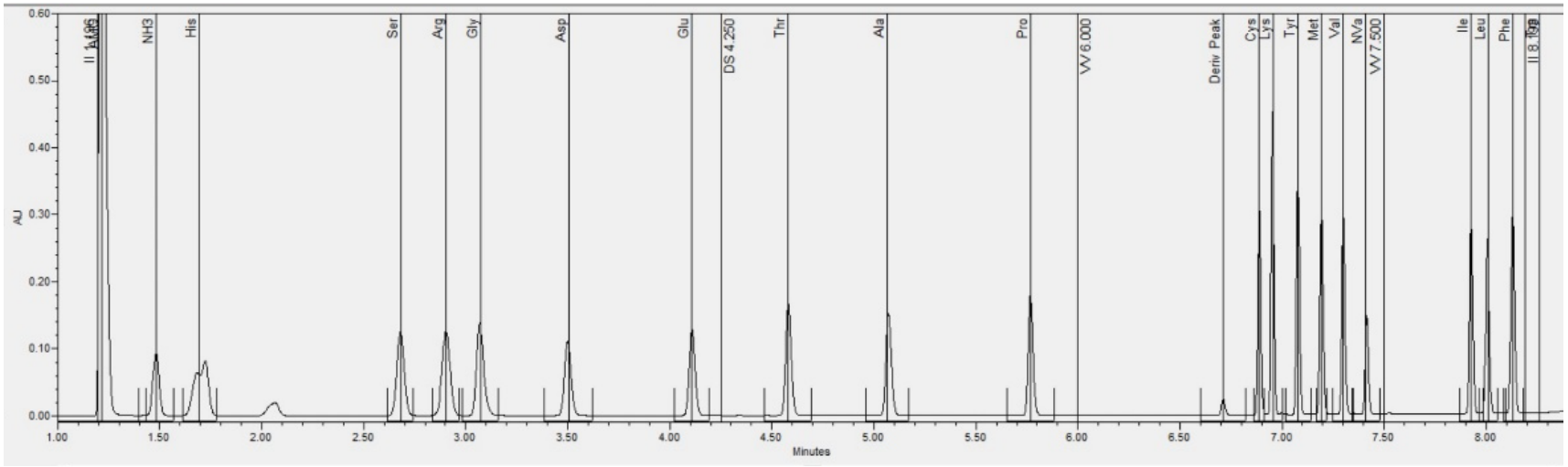


**Figure 8.** Chromatographic separation for the Amino Acid Standard H mixture on Waters Acquity UPLC-BEH C18 column.

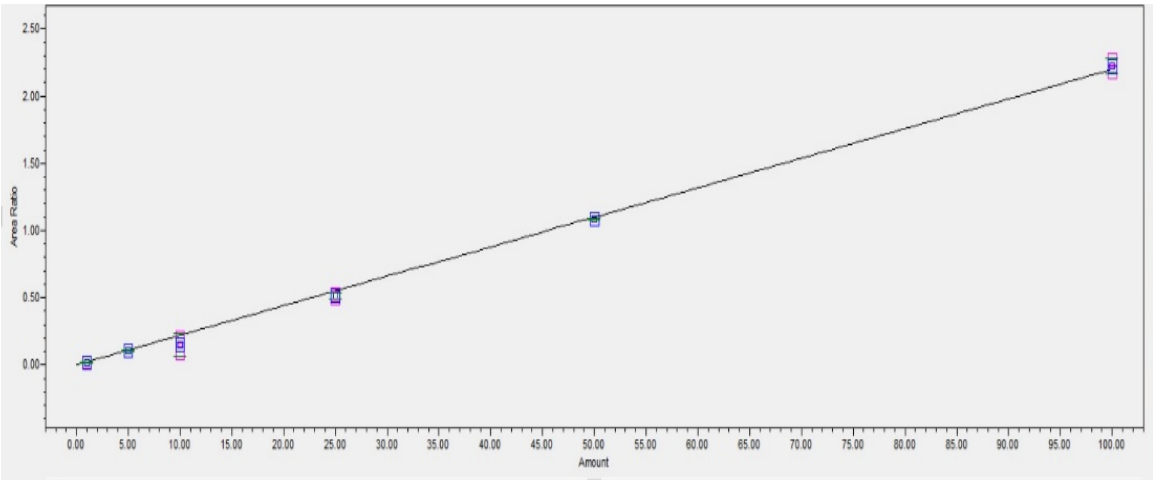


**Figure 9.** Calibration curve for arginine on Waters Acquity UPLC-BEH C18 column.

## Appendix I: Amino Acid Composition and Protein Digestibility

**Table 5.** Amino acid compositions (weight percentage, wet basis) of cPPI, nPPI, isolated fractions, reconstituted protein isolates, and legumin-enriched nPPIs with varying 7S to 11S ratios.

| Amino Acid | Weight percentage (%) amino acid in sample | | | | | | | | |
|---|---|---|---|---|---|---|---|---|---|
| | cPPI | nPPI | 100V | 80V-20L | 50V-50L | nPPI-50LE | 20V-80L | nPPI-80LE | 100L |
| L-Cysteine | 1.50 | 0.60 | 0.82 | 1.01 | 1.21 | 1.43 | 1.59 | 1.56 | 2.40 |
| L-Methionine | 0.74 | 1.14 | 0.69 | 0.59 | 0.71 | 1.60 | 0.90 | 1.46 | 1.47 |
| L-Tryptophan | 0.80 | 0.82 | 0.43 | 0.65 | 0.80 | 1.13 | 1.03 | 1.26 | 1.19 |
| L-HydroxyProline | 0.00 | 0.00 | 0.00 | 0.00 | 0.00 | 0.00 | 0.00 | 0.00 | 0.00 |
| L-Aspartic acid | 7.85 | 12.32 | 10.54 | 12.50 | 11.89 | 10.68 | 13.09 | 12.42 | 12.63 |
| L-Threonine | 1.70 | 2.43 | 1.62 | 2.19 | 2.21 | 2.15 | 2.23 | 2.50 | 2.52 |
| L-Serine | 4.39 | 4.33 | 4.44 | 4.70 | 4.79 | 4.22 | 4.60 | 4.43 | 4.40 |
| L-Glutamic acid | 20.44 | 22.89 | 18.21 | 21.45 | 21.10 | 19.94 | 21.34 | 22.66 | 23.58 |
| L-Proline | 4.60 | 4.10 | 3.23 | 3.84 | 4.09 | 3.73 | 3.98 | 4.27 | 4.13 |
| L-Glycine | 2.10 | 3.09 | 2.48 | 2.98 | 3.31 | 3.20 | 3.21 | 3.50 | 3.33 |
| L-Alanine | 2.69 | 3.53 | 2.29 | 3.04 | 3.12 | 3.35 | 3.15 | 3.73 | 3.72 |
| L-Valine | 2.48 | 2.97 | 2.57 | 3.27 | 3.18 | 2.59 | 3.08 | 3.21 | 3.19 |
| L-Isoleucine | 2.64 | 2.62 | 2.70 | 3.28 | 3.10 | 2.23 | 2.92 | 2.79 | 2.73 |
| L-Leucine | 5.31 | 6.58 | 7.39 | 7.86 | 7.88 | 6.40 | 7.44 | 7.09 | 6.99 |
| L-Tyrosine | 3.52 | 2.54 | 2.59 | 2.86 | 3.03 | 2.53 | 2.88 | 2.76 | 2.58 |
| L-Phenylalanine | 2.95 | 3.57 | 4.53 | 4.55 | 4.71 | 3.54 | 4.35 | 3.78 | 3.56 |
| L-Lysine | 5.36 | 5.91 | 5.77 | 6.38 | 5.90 | 5.25 | 5.65 | 5.58 | 5.48 |
| L-Histidine | 1.36 | 1.94 | 1.41 | 1.95 | 2.13 | 1.86 | 2.12 | 2.30 | 2.35 |
| L-Arginine | 3.61 | 7.91 | 5.66 | 7.73 | 8.29 | 7.36 | 11.87 | 9.06 | 9.57 |

**Table 6.** Amino acid score and *in vitro* protein digestibility (%) of cPPI, nPPI, isolated fractions, reconstituted protein isolates, and legumin-enriched nPPIs with varying 7S to 11S ratios.

| Sample | Amino Acid Score[1] | *In vitro* Protein Digestibility (%) |
|---|---|---|
| cPPI | 0.630[d2] | 1.08[d] |
| nPPI | 0.800[a] | 1.21[bc] |
| 100V | 0.3888[e] | 1.07[d] |
| 80V-20L | 0.594[d] | 1.17[c] |
| 50V-50L | 0.649[cd] | 1.20[bc] |
| nPPI-50LE | 0.632[d] | 1.17[c] |
| 20V-80L | 0.654[bcd] | 1.32[a] |
| nPPI-80LE | 0.736[abc] | 1.23[bc] |
| 100L | 0.742[ab] | 1.26[ab] |

[1]Calculated using the recommended amino acid scoring pattern for children (2 to 5 years) (FAO/WHO Expert Consultation, 1991)

[2]Means (n = 2) in each column with different lowercase letters indicate significant differences among samples according to the Tukey-Kramer multiple means comparison test ($P < 0.05$).

Sample calculation for amino acid score (AAS) and PDCAAS :

$$AAS = \frac{First\ limiting\ amino\ acid\ content\ of\ test\ protein}{First\ limiting\ amino\ acid\ content\ in\ reference\ amino\ acid\ pattern}$$

$$AAS\ (nPPI^{*}) = \frac{20.01 \frac{mg}{g}\ protein}{25.00\ \frac{mg}{g} protein^{**}} = 0.800$$

[*]L-Cysteinine + L-Methionine were identified as the first limiting amino acid in nPPI; 20.01 mg/g protein were detected

[**]1991 Reference Protein for L-Cysteinine + L-Methionineis 25.00 mg/g protein

$$PDCAAS = amino\ acid\ score\ of\ first\ limiting\ amino\ acid \times true\ digestibility$$

$$\text{PDCAAS (nPPI*)} = 0.800 \times 1.00 = 0.800$$

[*]Results >100% for *in vitro* digestibility were truncated down to 100% per the manufacturer's instructions

## Appendix J: ANOVA Tables

**Table 7.** Analysis of variance on the effect of sample on the area percentage of soluble aggregates present in samples analyzed by SE-HPLC.

| Sample Analysis | Source of Variation | Degrees of Freedom | Mean Square | F | Sig. |
|---|---|---|---|---|---|
| cPPI, nPPI, 100V, 80V-20L, 50V-50L, nPPI-50LE, 20V-80L, nPPI-80LE, 100L | Between Groups | 8 | 136.672 | 81.817 | 1.70e-07 |
| | Error | 9 | 1.670 | | |

**Table 8.** Analysis of variance on the effect of sample on the area percentage of legumin present in samples analyzed by SE-HPLC.

| Sample Analysis | Source of Variation | Degrees of Freedom | Mean Square | F | Sig. |
|---|---|---|---|---|---|
| cPPI, nPPI, 100V, 80V-20L, 50V-50L, nPPI-50LE, 20V-80L, nPPI-80LE, 100L | Between Groups | 8 | 523.582 | 3389.915 | 9.85e-15 |
| | Error | 9 | 1.390 | | |

**Table 9.** Analysis of variance on the effect of sample on the area percentage of *con*vicilin present in samples analyzed by SE-HPLC.

| Sample Analysis | Source of Variation | Degrees of Freedom | Mean Square | F | Sig. |
|---|---|---|---|---|---|
| cPPI, nPPI, 100V, 80V-20L, 50V-50L, nPPI-50LE, 20V-80L, nPPI-80LE, 100L | Between Groups | 8 | 3.237 | 42.767 | 2.89e-06 |
| | Error | 9 | 0.076 | | |

**Table 10.** Analysis of variance on the effect of sample on the area percentage of vicilin present in samples analyzed by SE-HPLC.

| Sample Analysis | Source of Variation | Degrees of Freedom | Mean Square | F | Sig. |
|---|---|---|---|---|---|
| cPPI, nPPI, 100V, 80V-20L, 50V-50L, nPPI-50LE, 20V-80L, nPPI-80LE, 100L | Between Groups | 8 | 436.652 | 1221.072 | 9.71e-13 |
| | Error | 9 | 0.358 | | |

**Table 11.** Analysis of variance on the effect of sample on the area percentage of soluble aggregates present in samples dissolved in 0.1% SDS phosphate buffer and analyzed by SE-HPLC.

| Sample Analysis | Source of Variation | Degrees of Freedom | Mean Square | F | Sig. |
|---|---|---|---|---|---|
| cPPI, nPPI, 100V, 80V-20L, 50V-50L, nPPI-50LE, 20V-80L, nPPI-80LE, 100L | Between Groups | 8 | 149.457 | 531.845 | 4.05e-11 |
| | Error | 9 | 0.281 | | |

**Table 12**. Analysis of variance on the effect of sample on the area percentage of legumin present in samples dissolved in 0.1% SDS phosphate buffer and analyzed by SE-HPLC.

| Sample Analysis | Source of Variation | Degrees of Freedom | Mean Square | F | Sig. |
|---|---|---|---|---|---|
| cPPI, nPPI, 100V, 80V-20L, 50V-50L, nPPI-50LE, 20V-80L, nPPI-80LE, 100L | Between Groups | 8 | 248.503 | 3960.422 | 4.89e-15 |
| | Error | 9 | 0.063 | | |

**Table 13.** Analysis of variance on the effect of sample on the area percentage of convicilin present in samples dissolved in 0.1% SDS phosphate buffer and analyzed by SE-HPLC.

| Sample Analysis | Source of Variation | Degrees of Freedom | Mean Square | F | Sig. |
|---|---|---|---|---|---|
| cPPI, nPPI, 100V, 80V-20L, 50V-50L, nPPI-50LE, 20V-80L, nPPI-80LE, 100L | Between Groups | 8 | 6.101 | 180.779 | 5.06e-09 |
| | Error | 9 | 0.034 | | |

**Table 14.** Analysis of variance on the effect of sample on the area percentage of vicilin present in samples dissolved in 0.1% SDS phosphate buffer and analyzed by SE-HPLC.

| Sample Analysis | Source of Variation | Degrees of Freedom | Mean Square | F | Sig. |
|---|---|---|---|---|---|
| cPPI, nPPI, 100V, 80V-20L, 50V-50L, nPPI-50LE, 20V-80L, nPPI-80LE, 100L | Between Groups | 8 | 454.529 | 11481.935 | 4.07e-17 |
| | Error | 9 | 0.040 | | |

**Table 15.** Analysis of variance on the effect of sample on the area percentage of soluble aggregates present in samples dissolved in 0.1% SDS + 2.5% BME phosphate buffer and analyzed by SE-HPLC.

| Sample Analysis | Source of Variation | Degrees of Freedom | Mean Square | F | Sig. |
|---|---|---|---|---|---|
| cPPI, nPPI, 100V, 80V-20L, 50V-50L, nPPI-50LE, 20V-80L, nPPI-80LE, 100L | Between Groups | 8 | 169.441 | 569.618 | 2.98e-11 |
| | Error | 9 | 0.297 | | |

**Table 16.** Analysis of variance on the effect of sample on the area percentage of legumin present in samples dissolved in 0.1% SDS + 2.5% BME phosphate buffer and analyzed by SE-HPLC.

| Sample Analysis | Source of Variation | Degrees of Freedom | Mean Square | F | Sig. |
|---|---|---|---|---|---|
| cPPI, nPPI, 100V, 80V-20L, 50V-50L, nPPI-50LE, 20V-80L, nPPI-80LE, 100L | Between Groups | 8 | 251.457 | 1474.243 | 4.16e-13 |
| | Error | 9 | 0.171 | | |

**Table 17.** Analysis of variance on the effect of sample on the area percentage of convicilin present in samples dissolved in 0.1% SDS + 2.5% BME phosphate buffer and analyzed by SE-HPLC.

| Sample Analysis | Source of Variation | Degrees of Freedom | Mean Square | F | Sig. |
|---|---|---|---|---|---|
| cPPI, nPPI, 100V, 80V-20L, 50V-50L, nPPI-50LE, 20V-80L, nPPI-80LE, 100L | Between Groups | 8 | 8.978 | 47.471 | 1.84e-06 |
| | Error | 9 | 0.189 | | |

**Table 18.** Analysis of variance on the effect of sample on the area percentage of vicilin present in samples dissolved in 0.1% SDS + 2.5% BME phosphate buffer and analyzed by SE-HPLC.

| Sample Analysis | Source of Variation | Degrees of Freedom | Mean Square | F | Sig. |
|---|---|---|---|---|---|
| cPPI, nPPI, 100V, 80V-20L, 50V-50L, nPPI-50LE, 20V-80L, nPPI-80LE, 100L | Between Groups | 8 | 275.174 | 211.705 | 2.50e-09 |
| | Error | 9 | 1.300 | | |

**Table 19.** Analysis of variance on the effect of sample on the thermal denaturation temperature of the DSC peak.

| Sample Analysis | Source of Variation | Degrees of Freedom | Mean Square | F | Sig. |
|---|---|---|---|---|---|
| cSPI*, cPPI*, nPPI, 100V, 100V-salt, 80V-20L, 50V-50L, nPPI-50LE, 20V-80L, nPPI-80LE, 100L, 100L-salt | Between Groups | 9 | 51.216 | 217.298 | 5.99e-18 |
| | Error | 20 | 0.236 | | |

*cSPI and cPPI were completely denatured before analysis. No peak of denaturation observed.

**Table 20.** Analysis of variance on the effect of sample on the enthalpy of denaturation of the DSC peak.

| Sample Analysis | Source of Variation | Degrees of Freedom | Mean Square | F | Sig. |
|---|---|---|---|---|---|
| cSPI*, cPPI*, nPPI, 100V, 100V-salt, 80V-20L, 50V-50L, nPPI-50LE, 20V-80L, nPPI-80LE, 100L, 100L-salt | Between Groups | 9 | 56.718 | 92.992 | 2.43e-14 |
| | Error | 20 | 0.610 | | |

*cSPI and cPPI were completely denatured before analysis. No peak of denaturation observed.

**Table 21.** Analysis of variance on the effect of sample on surface hydrophobicity.

| Sample Analysis | Source of Variation | Degrees of Freedom | Mean Square | F | Sig. |
|---|---|---|---|---|---|
| cSPI, cPPI, nPPI, 100V, 80V-20L, 50V-50L, nPPI-50LE, 20V-80L, nPPI-80LE, 100L | Between Groups | 11 | 21084278 | 118.460 | 2.34e-31 |
| | Error | 49 | 177986 | | |

**Table 22.** Analysis of variance on the effect of sample on surface charge.

| Sample Analysis | Source of Variation | Degrees of Freedom | Mean Square | F | Sig. |
|---|---|---|---|---|---|
| cSPI, cPPI, nPPI, 100V, 100V-salt, 80V-20L, 50V-50L, nPPI-50LE, 20V-80L, nPPI-80LE, 100L, 100L-salt | Between Groups | 11 | 1269.764 | 462.356 | 1.01e-81 |
| | Error | 102 | 2.746 | | |

**Table 23.** Analysis of variance on the effect of sample on solubility at pH 7.

| Sample Analysis | Source of Variation | Degrees of Freedom | Mean Square | F | Sig. |
|---|---|---|---|---|---|
| cSPI, cPPI, nPPI, 100V, 100V-salt, 80V-20L, 50V-50L, nPPI-50LE, 20V-80L, nPPI-80LE, 100L, 100L-salt | Between Groups | 11 | 1031.966 | 99.733 | 4.75e-23 |
| | Error | 35 | 10.347 | | |

**Table 24.** Analysis of variance on the effect of sample on solubility at pH 7 and heated at 80°C.

| Sample Analysis | Source of Variation | Degrees of Freedom | Mean Square | F | Sig. |
|---|---|---|---|---|---|
| cSPI, cPPI, nPPI, 100V, 100V-salt, 80V-20L, 50V-50L, nPPI-50LE, 20V-80L, nPPI-80LE, 100L, 100L-salt | Between Groups | 11 | 419.116 | 46.394 | 3.26e-17 |
| | Error | 34 | 9.034 | | |

**Table 25.** Analysis of variance on the effect of sample on solubility at pH 3.4.

| Sample Analysis | Source of Variation | Degrees of Freedom | Mean Square | F | Sig. |
|---|---|---|---|---|---|
| cSPI, cPPI, nPPI, 100V, 100V-salt, 80V-20L, 50V-50L, nPPI-50LE, 20V-80L, nPPI-80LE, 100L, 100L-salt | Between Groups | 11 | 2002.229 | 582.407 | 1.81e-34 |
| | Error | 33 | 3.438 | | |

**Table 26.** Analysis of variance on the effect of sample on solubility at pH 3.4 and heated at 80°C.

| Sample Analysis | Source of Variation | Degrees of Freedom | Mean Square | F | Sig. |
|---|---|---|---|---|---|
| cSPI, cPPI, nPPI, 100V, 100V-salt, 80V-20L, 50V-50L, nPPI-50LE, 20V-80L, nPPI-80LE, 100L, 100L-salt | Between Groups | 11 | 4528.641 | 722.653 | 6.73e-37 |
| | Error | 34 | 6.267 | | |

**Table 27.** Analysis of variance on the effect of sample on gel strength at 20% protein concentration.

| Sample Analysis | Source of Variation | Degrees of Freedom | Mean Square | F | Sig. |
|---|---|---|---|---|---|
| cSPI*, cPPI, nPPI, 100V, 100V-salt, 80V-20L, 50V-50L, nPPI-50LE**, 20V-80L, nPPI-80LE, 100L, 100L-salt | Between Groups | 10 | 213.227 | 899.250 | 1.51e-54 |
| | Error | 52 | 0.237 | | |

*Gel strength was measured at 15% protein concentration for cSPI
**No gel formed

**Table 28.** Analysis of variance on the effect of sample on emulsification capacity.

| Sample Analysis | Source of Variation | Degrees of Freedom | Mean Square | F | Sig. |
|---|---|---|---|---|---|
| cSPI, cPPI, nPPI, 100V, 100V-salt, 80V-20L, 50V-50L, nPPI-50LE, 20V-80L, nPPI-80LE, 100L, 100L-salt | Between Groups | 11 | 183086 | 125.443 | 1.98e-18 |
| | Error | 24 | 1459.519 | | |

**Table 29.** Analysis of variance on the effect of sample on the percentage (g AA/ 100 g sample) of sulfur-containing amino acids.

| Sample Analysis | Source of Variation | Degrees of Freedom | Mean Square | F | Sig. |
|---|---|---|---|---|---|
| cPPI, nPPI, 100V, 80V-20L, 50V-50L, nPPI-50LE, 20V-80L, nPPI-80LE, 100L | Between Groups | 8 | 1.864 | 139.922 | 5.63e-14 |
| | Error | 17 | 0.013 | | |

**Table 30.** Analysis of variance on the effect of sample on the percentage (g AA/ 100 g sample) of acidic amino acids.

| Sample Analysis | Source of Variation | Degrees of Freedom | Mean Square | F | Sig. |
|---|---|---|---|---|---|
| cPPI, nPPI, 100V, 80V-20L, 50V-50L, nPPI-50LE, 20V-80L, nPPI-80LE, 100L | Between Groups | 8 | 23.804 | 18.345 | 6.20e-07 |
| | Error | 17 | 1.298 | | |

**Table 31.** Analysis of variance on the effect of sample on the percentage (g AA/ 100 g sample) of basic amino acids.

| Sample Analysis | Source of Variation | Degrees of Freedom | Mean Square | F | Sig. |
|---|---|---|---|---|---|
| cPPI, nPPI, 100V, 80V-20L, 50V-50L, nPPI-50LE, 20V-80L, nPPI-80LE, 100L | Between Groups | 8 | 15.241 | 47.856 | 3.75e-10 |
| | Error | 17 | 0.318 | | |

**Table 32.** Analysis of variance on the effect of sample on the ratio of acidic to basic amino acids.

| Sample Analysis | Source of Variation | Degrees of Freedom | Mean Square | F | Sig. |
|---|---|---|---|---|---|
| cPPI, nPPI, 100V, 80V-20L, 50V-50L, nPPI-50LE, 20V-80L, nPPI-80LE, 100L | Between Groups | 8 | 0.146 | 21.404 | 1.98e-07 |
| | Error | 17 | 0.007 | | |

**Table 33.** Analysis of variance on the effect of sample on the percentage (g AA/ 100 g sample) of hydrophobic amino acids.

| Sample Analysis | Source of Variation | Degrees of Freedom | Mean Square | F | Sig. |
|---|---|---|---|---|---|
| cPPI, nPPI, 100V, 80V-20L, 50V-50L, nPPI-50LE, 20V-80L, nPPI-80LE, 100L | Between Groups | 8 | 10.075 | 1.865 | 0.1334 |
| | Error | 17 | 5.403 | | |

**Table 34.** Analysis of variance on the effect of sample on the percentage (g AA/ 100 g sample) of critical amino acids.

| Sample Analysis | Source of Variation | Degrees of Freedom | Mean Square | F | Sig. |
|---|---|---|---|---|---|
| cPPI, nPPI, 100V, 80V-20L, 50V-50L, nPPI-50LE, 20V-80L, nPPI-80LE, 100L | Between Groups | 8 | 10.038 | 4.770 | 0.0033 |
| | Error | 17 | 2.105 | | |

**Table 35.** Analysis of variance on the effect of sample on protein digestibility**.**

| Sample Analysis | Source of Variation | Degrees of Freedom | Mean Square | F | Sig. |
|---|---|---|---|---|---|
| cPPI, nPPI, 100V, 80V-20L, 50V-50L, nPPI-50LE, 20V-80L, nPPI-80LE, 100L | Between Groups | 8 | 0.013 | 42.439 | 2.996e-06 |
| | Error | 9 | 0.00032 | | |