WET FRACTIONATION APPROACHES FOR ISOLATING PULSE PROTEINS AND THEIR MODIFICATION THROUGH PH SHIFTING

A Thesis Submitted to the

College of Graduate and Postdoctoral Studies

in Partial Fulfillment of the Requirements for the

Degree of Doctor of Philosophy in the

Department of Food and Bioproduct Sciences

University of Saskatchewan

Saskatoon, Saskatchewan, Canada

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2024

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ABSTRACT

Plant protein extraction involves separating proteins from other plant materials. The extracted proteins are then used in various food and non-food applications depending on their nutritional and functional properties. In the case of food ingredients, markets require consistent and reliable protein ingredients. Pulses are high in proteins, and their extracted proteins exhibit good functionality when used as ingredients, such as foaming or emulsifying agents. However, the processing conditions to which the proteins are subject can impact the protein structure and affect their functionality. The overarching goal of this study was to investigate the impact of extraction conditions, such as flour to water ratio (f:w), particle size of the starting flours and the use of different extraction methods, on the surface and functional properties of four different pulses: yellow peas (YP), green lentils (GL), kabuli chickpeas (CH) and navy beans (NB). Additionally, the effect of a pH shifting method was investigated on a commercial pea protein as a model for a pulse protein, with the aim of enhancing pulse protein solubility, foaming and emulsifying properties.

The first study (Chapter 3) examined the effect of reducing water levels used during alkaline extraction, where three different flour:water ratios were tested (1:10, which is the most commonly used ratio, 1:7 and 1:5) on fine pulse flours (sieved through a 0.5 mm mesh size screen, P1) and the resulting extraction, protein yields, and functionality were assessed. The results showed that reducing the water levels used in the extraction process did not result in changes in functionality; however, it led to a significant decrease in the protein yields recovered. Due to these protein losses, a 1:10 f:w ratio was selected to perform extractions with a coarse flour (1.27 mm mesh size screen, P2) to assess extraction yields as well as functionality. Results showed that using a fine flour led to an increase in both extraction (0.4 to 3.2 % increase) and protein yields (7.9 to 10.3 % increase). In terms of the functional properties, P1 and P2 extracted proteins differed in some functional properties, but these differences were not consistent for all the pulses evaluated. Determination of the legumin (L) and vicilin (V) ratios of the pulse types evaluated led to finding differences in functionality between the different pulse types tested: NB exhibited the highest solubility and emulsifying properties, while CH showed the highest foaming capacity (FC). This study highlights the impact of milling and control of the particle size of the starting flours on the extraction of pulse proteins.

The second study (Chapter 4) focused on the effect of using different extraction methods, alkaline extraction – isoelectric precipitation - AEIP and salt extraction- SE, on the surface, functional and nutritional properties of protein isolates extracted from the same pulses tested in the first study. The optimum conditions found in the first study were used in this study as well since the 1:10 f:w ratio and P1 flours achieved the highest yields. Results showed that the extraction method influences the type of proteins being extracted, impacting the albumin (A)/ globulin (G) ratios. The SE-extracted proteins extracted a greater proportion of albumins than the AEIP method, and these differences in protein composition had an effect on the nutritional properties as well as on protein functionality, impacting protein solubility, foaming and emulsifying properties. The proteins extracted using an AEIP method showed increased *in-vitro* protein digestibility (IVPD), while the SE proteins showed overall increased functionality over the AEIP proteins.

The last study (Chapter 5) explored the effect of acid (pH 2) and alkaline (pH 10) pH shifting in combination with temperature on a commercial pea protein isolate as a modification method to improve the protein's solubility, foaming and emulsifying properties. Results from this study indicated that an alkaline pH shifting, as well as in combination with heat, led to increases in protein solubility, while an acidic treatment as well as in combination with heat negatively impacted this functional property. The FC of the samples was increased by an alkaline pH shifting, while FS results were either neutral or negatively impacted by the treated samples. An analysis of the foam structure using a dynamic foam analyzer (DFA) allowed us to explore differences and to monitor the bubble structure of the resulting foams.

Overall, this research explored the effects of protein extraction methods and conditions on the resulting functionality and nutritional properties of common pulses grown in Canada. Knowledge from these studies can provide further understanding on the impact of changes in protein structure during processing and aid in obtaining consistent ingredients with the expected functionality for food applications.

ACKNOWLEDGEMENTS

I would like to express my sincere appreciation and gratitude to Dr. Michael T. Nickerson for his guidance, support, and motivating encouragement. I appreciate Dr. Nickerson's sense of humor as well as the diverse learning opportunities and the nurturing environment he provided, fostering both personal and academic growth.

I would like to thank my external examiner Dr. Lingyun Chen from the University of Alberta, committee members and graduate advisory committee, Drs. Tom Warkentin, Takuji Tanaka, Supratim Ghosh and Christopher Eskiw for their support, insightful comments, corrections and thought-provoking questions. Their collective guidance has significantly contributed to the advancement of my academic pursuits.

I extend my gratitude to several members of the Food and Bioproduct Sciences Department, including Yuanlong Cao for his assistance and help with all lab-related matters, and to Donna Selby, Ann Harley and Shahrima Tasin for their valuable administrative support.

I would like to thank Andrea Stone, Dellaney Konieczny, Dai Shi and Lindsay Wang. Thank you for dedicating your time and sharing your knowledge.

I am thankful to friends and fellow lab members Cassia Galves, Maria Guerrero, Monica Mogrovejo, CJ Manning, Joy Sareen, and many others for the time we shared and for making me laugh.

Lastly, I would like to express my heartfelt appreciation to my family. My parents for always being supportive of all my ideas and plans, my husband, Herman, for being an enthusiastic cheerleader in everything I do, and my friends back home for their encouragement and unconditional support. I am grateful for their love and support.

Financial support for this research was provided through 1) the Agriculture and Agri-Food Canada's AgriScience Program (Canadian Pulse Science Research Cluster) under the Canadian Agricultural Partnership, the Saskatchewan Pulse Growers, the Alberta Pulse Growers, the Manitoba Pulse and Soybean Growers; 2) the Natural Sciences and Engineering Research Council of Canada (NSERC) Discovery grant program; and 3) the NSERC CREATE – CAPTURE program.

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LIST OF ABBREVIATIONS AND SYMBOLS

AA	Amino acids
A	Albumin
AEIP	Alkaline extraction isoelectric precipitation
ANF	Anti-nutritional factors
ANOVA	Analysis of Variance
ANS	8-anilino-1-naphtalensulfonic acid
CH	Chickpea
CIGI	Canadian International Grains Institute
D_{32}	Area average mean diameter
d.b.	Dry basis
DIAAS	Digestible indispensable amino acid score
V	Vicilin
EC	Emulsion capacity
EAI	Emulsion activity index
ESI	Emulsion stability index
FC	Foam capacity
FI	Fluorescence intensity
FS	Foam stability
G	Globulin
GL	Green lentil
IVPD	In-vitro protein digestibility
IV-PDCAAS	In vitro protein digestibility corrected amino-acid score
L	Legumin
MP	Micellar precipitation
Mw	Molecular weight
NB	Navy bean
OHC	Oil holding capacity
PAGE	Polyacrylamide gel electrophoresis
PDCAAS	Protein digestibility corrected amino acid score

PER	Protein efficiency ratio
PR	Protein rating
$R_{3,2}$	Sauter mean radius
GL	Green lentil
S	Sedimentation coefficient
SDS	Sodium dodecyl sulphate
SE	Salt extraction
UV	Ultraviolet
VIS	Visible
WHC	Water holding capacity
YP	Yellow pea
w:f	water:flour ratio
λ	Wavelength
ζ	Zeta potential
ε	Permittivity
κ	Particle radius
α	Debye length
η	Viscosity
φ	Volume fraction
γ	Interfacial tension

1. INTRODUCTION

1.1. Overview

Cereals, oilseeds, and pulses are the most important crops used as food and feed around the globe. Pulses, particularly, are higher in protein than cereals (~20%-30%) and are harvested for their dried seeds (Bessada *et al.*, 2019). The pulses commonly grown in Canada are field peas, lentils, beans, and chickpeas. These pulses are commercialized whole, split, processed into flours, or their proteins are extracted using different methods and then applied to many different products (Siddiq & Uebersax, 2012). Due to the growing demand for plant-based protein sources, legumes are an attractive group of crops to fulfill these demands, since their production requires fewer resources than animal protein sources, including meat and dairy (Bessada *et al.*, 2019).

The main proteins found in pulses are globulins (salt-soluble) and albumins (water-soluble), constituting approximately 70% to 80% and 10% to 20% of the total proteins, respectively (Lam *et al.*, 2018). The globulin fraction is mainly comprised of 11*S* legumins and 7*S* vicilins. The ratios of legumins/ and vicilins can be found ranging from 0.4 to 4.2, depending on the pulse type and cultivar (Lam *et al.*, 2018, Swanson 1990). Legumins are hexameric proteins with subunits linked by disulphide bonds. They are richer in cysteine and methionine than vicilins. Vicilins are trimeric proteins with subunits held together by hydrophobic interactions (Derbyshire *et al.*, 1976). These proteins have different sizes and amino acidic composition, which, in turn, influence their structure and behaviour when they are tested for their functionality (Shen & Tang, 2014). The ratio at which legumins and vicilins are found in pulses depends on different factors such as the pulse variety, crop year, environmental conditions and cultivar (Lam *et al.*, 2017; Stone *et al.*, 2015a), therefore will also determine how these proteins will perform when used as ingredients. Albumins are small proteins (~<20 kDa) and can also impact the functionality given that they are water-soluble and have higher flexibility allowing them to act as good surface-active components (Osemwota *et al.*, 2022). The method of extraction used can also influence the ratio of albumins and globulins found in protein isolates and concentrates, given the protein's differences in solubilities (Stone *et al.*, 2015b). Among the extraction methods commonly used for protein extraction, dry extraction

methods are more energy and resource-efficient; however, the protein purity achieved (40-75%) is lower than the obtained with wet extraction methods (>90%) because the fine fraction containing the proteins is contaminated with the coarser starch-rich fraction (Singhal *et al.*, 2016). On the other hand, wet extraction methods use the solubilization of the proteins (through pH adjustment of the proteins or addition of salts) to separate them from other components, and this selective step could impact the type of proteins being extracted (Stone *et al.*, 2015b). However, wet extraction methods use large amounts of water and need a higher energy input since the extracted proteins need a posterior drying step.

Therefore, understanding the impact of extraction conditions and methods on the extraction and protein yields, the type of proteins being extracted, the nutritional attributes, and the resulting functionality is crucial. These aspects are essential for processing optimization and production of proteins with customized functionality specifically tailored for applications in the food industry. Furthermore, knowing that animal-based proteins tend to outperform plant-based proteins due to increased flexibility and smaller size leading to improved functionality, this highlights the importance of researching ways to modify plant proteins. This investigation can be essential for improving the functionality and application of plant proteins in food (e.g., baking, beverages, nutritional/sport products, meat analogs, etc.) and non-food (e.g., bio-adhesives, bio-packaging and bio-plastics) sectors.

The overall goal of this thesis research was to study extraction conditions and protein modification through pH shifting on the surface and functional properties of four pulses commonly grown in Canada (green lentils (GL), kabuli chickpeas (CH), navy beans (NB) and yellow peas (YP)). The extraction conditions studied included water reduction in the wet extraction process of proteins, the use of flours of different particle sizes, as well as two different extraction methods (salt extraction (SE) and alkaline extraction – isoelectric precipitation (AEIP)). In addition, the effects of modification of a commercial pea protein sample through acidic and alkaline pH shifting were also performed.

1.2. Hypotheses

a) A water-to-flour variation will affect protein extraction yields.

b) Smaller particle-sized flours will favour protein extraction, resulting in proteins with increased functionality compared to those obtained from coarser flours, due to their large surface area and more efficient extraction.
c) Differences in functional properties will be found among different pulse types.
d) The extraction method will be an influential factor in the yield of the extracted proteins.
e) The protein extraction method will influence the physicochemical and functional properties of the protein isolates due to the extraction of different proteins with each extraction technique.
f) pH shifting will increase the solubility and functionality of a commercial pea protein isolate.
g) Combining a pH-shifting method and heating will further influence the changes occurring at the protein's surface, potentially promoting increased functionality.

1.3. Objectives

To evaluate these hypotheses, the following objectives will be assessed:

a) To use an AEIP extraction method with three different f:w ratios to evaluate the effect of reducing water on the protein extraction yield.
b) Extract proteins using an AEIP method and assess their yields, surface, and functional properties using flours sieved through two different mesh sizes.
c) Extract proteins using SE and AEIP extraction methods to evaluate differences in their yields, surface, and functional properties.
d) Use a pH shifting method in combination with heat to prepare alkaline (pH 10) and acidic (pH 2) samples to evaluate their surface, foaming and emulsifying properties.

2. LITERATURE REVIEW

2.1. Overview of pulses

Pulses (navy beans, yellow peas, green lentils, and kabuli chickpeas), pertaining to the legume family, are harvested for their dry seeds and constitute, along with cereals, one of the major sources of food crops around the world (Rebello *et al.*, 2014). Western Canada is one of the largest producers and exporters of these crops. They have been included in cereal and oilseeds crop rotations due to their Nitrogen fixation ability through their symbiosis with rhizobacteria, leading to a reduction in the use of fertilizers, as well as other advantages like reducing pest control, which in turn, lead to economic and environmental benefits (MacWilliam *et al.*, 2014). While protein content of cereals ranges between 10% to 15% (dry basis, d.b.), pulses have a protein content ranging from 20% to 30% (d.b.). They are high on aspartic and glutamic acid, leucine, arginine and lysine but low on tryptophan and the sulphur-containing amino acids cysteine and methionine, which are complemented by cereals if they are consumed together (Nosworthy & House, 2017; Rebello *et al.*, 2014). Because of changing dietary patterns, rising costs of animal proteins, environmental concerns, and increasing demand for nutritious and healthier options, consumers demand proteins from a broader range of alternative sources (Fasolin *et al.*, 2019; McClements & Grossmann, 2021). Therefore, knowledge of the functional attributes of protein-rich fractions is essential to better incorporate them into our foods. These properties include solubility, water and oil holding capacities, emulsifying and foaming properties, which allow pulse ingredients to be incorporated into different applications such as pastas or baked products (Boukid *et al.*, 2019), meat products (Pathiraje *et al.*, 2023), spreads and snacks among others (Asif *et al.*, 2013).

Pulses have a protein content of about 20%-30%, and are found in the protein bodies of the seeds. These proteins are called storage proteins since the plant will use them as a nitrogen source for later growth and use during germination (Derbyshire *et al.*, 1976). The proteins present in pulses are mainly globulin- and albumin-type proteins, representing approximately 60%-80% and 10%-20% of the total protein, respectively (Yang *et al.*, 2022). Other proteins like prolamins or glutelins are also present but in smaller amounts. According to the Osborne classification of

proteins, albumins are soluble in water and are richer in threonine, tryptophan and lysine amino acids. Globulins are soluble in salt-water solutions and can be grouped into legumins (hexameric proteins with a sedimentation coefficient (*S*) of 11*S* and molecular weight (Mw) of ~ 340 – 400 kDa), vicilins (trimeric proteins, 7*S* and Mw of ~ 150 kDa) and convicilins found in lower amounts (7S and Mw of ~210 kDa) (Yang *et al.*, 2021). Legumin and convicilin have sulphur-containing amino acids and a lower glutamic/aspartic acid ratio. Vicilin does not contain cysteine or methionine and has a higher glutamic/ aspartic acid ratio (Croy *et al.*, 1980). The ratio of legumin to vicilin (L/V ratio) varies depending on pulse variety, seed development, agronomic practices and growth conditions (Lam *et al.*, 2017). Martinez *et al.* (2016) studied the L/V ratio of faba beans from two locations and examined whether this ratio was affected by genotype and environment. The authors found that the L/V ratio was significantly affected by environment, while genotype or the interaction between these two factors did not affect the L/V content. Lam *et al.* (2017) found no effect of the environmental conditions on L/V ratios when studying different pea cultivars from two growing seasons. However, a significant difference among the L/V ratios of different cultivars was found. No interaction between environment and cultivar was found to influence the protein ratios.

The difference in L/V ratios in different pulses and pulse varieties can influence the functional properties of protein isolates or protein concentrates. For example, higher legumin content could lead to lower emulsifying properties since its structure is more rigid and undergoes conformational changes at interfaces less effectively than vicilins (Shi & Nickerson, 2022).

2.2. Protein extraction

The extraction of proteins can be carried out using dry or wet extraction methods. Dry extraction is considered a more sustainable method since it does not use water and uses less energy since no drying step is involved. The lack of a drying step is also beneficial for retaining the native structure of the proteins. This method separates proteins (fine fraction) from starch (coarse fraction) from milled pulses by air classification. However, the protein content achieved ranges from 45%-70% and is of lesser purity than those achieved by wet extraction (Schutyser *et al.*, 2015). Wet extraction methods include alkaline extraction followed by isoelectric precipitation (AEIP) or ultra-filtration (UF), salt extraction (SE) followed by dialysis or micellar precipitation (MP) (Lam *et al.*, 2018) to achieve protein concentrates (>70%) or protein isolates (>90%). Wet

extractions typically start with the dry milling of the dehulled seeds and then the defatting of the flour, depending on the oil content of the pulse. The oil content of lupin or chickpeas is higher than other pulses (up to 13%), but for most pulses, it is usually between 1-3% (Hall *et al.*, 2017). This defatting process can be done using organic solvents, such as hexane (Can Karaca *et al.*, 2011b), ethanol, acetone, petroleum ether or chloroform (Feyzi *et al.*, 2017). Defatting of the flours can inhibit emulsion formation during the wet extraction process and improve protein solubility since there would be fewer protein-lipid interactions in the extracted proteins (Galves *et al.*, 2019).

The following sections will present common methods and factors influencing protein extraction.

2.3. Protein wet extraction methods

The wet extraction of proteins consists of solubilizing the proteins to separate them from other insoluble components, followed by precipitation of the proteins by adjusting the conditions of the extraction solvent to promote protein-protein interactions. The solvent in which the proteins are solubilized is usually water. However, adding salts, acids, or bases increases protein solubility, favouring protein-water interactions.

2.4. Alkaline extraction - isoelectric precipitation

This extraction method is one of the most used for protein extraction due to the high yields that can be obtained compared to other methods. Alkaline extraction – isoelectric precipitation (AEIP) makes use of the protein's high solubility at alkaline pH. Proteins carry a negative charge at this pH (pH 8.0-9.0), so the interactions between protein and water are favored over protein-protein ones since the negative charges create electrostatic repulsions between proteins, remaining in solution. Once the proteins are solubilized, separation of the proteins from the insoluble fractions is achieved by centrifugation. Following this step, the pH of the supernatant is adjusted to the isoelectric pH (~4.5 for pulses), where proteins take on a neutral net charge, and the interactions between proteins are favored, causing them to fall out of the solution and precipitate. The proteins are then centrifuged to collect them in the pellet, washed and dried using different methods, such as freeze-drying or spray-drying (Lam *et al.*, 2017; Singhal *et al.*, 2016).

After alkaline extraction, ultrafiltration or diafiltration of the supernatant containing the solubilized proteins can be used instead of isoelectric precipitation to collect the proteins. This

method employs various pore-size membranes to separate proteins based on their molecular weight, also helping in the removal of oligosaccharides (Fredrikson *et al.*, 2001; Singhal *et al.*, 2016). Alonso-Miravalles *et al.* (2019) studied the protein extraction of lentils using both AEIP and UF. They found that the protein content obtained in AEIP (85.1%) was significantly lower than the one obtained through ultrafiltration (93.7%). The proteins collected using the latter method also showed better functional properties. This improvement in functional properties or protein yield is mainly attributed to gentler conditions offering higher selectivity due to the selection of pore size and extracting conditions.

2.5. Salt extraction (SE) – Micellar precipitation (MP)

Adding salt to a protein solution can favor protein-water or protein-protein interactions depending on the concentration and type of salt used. The selection of salt for protein extraction depends on the ion's ability to solubilize or precipitate proteins according to the Hofmeister series (Shimizu *et al*., 2006). At low salt concentrations (*e.g*., NaCl), protein-water interactions are favored as the salt helps to structure the hydration layer surrounding the proteins. This process is known as salting-in. Conversely, salting-out happens at higher concentrations of salts or when selecting salting-out type salts (*e.g.*, ammonium sulphate). In this process, the presence of salt favors ion-water interactions over protein-water interactions, leading to the disruption of the hydration layers surrounding the proteins. As a result, hydrophobic amino acids at the surface of the proteins will favor protein-protein interactions and fall out of the solution (Singhal *et al.*, 2016). According to the Osborne classification scheme of protein solubilities, this extraction method can separate the major proteins present in pulses, albumins (water soluble) and globulins (salt soluble). With the addition of salt, the solubilized proteins are found in the supernatant. They can be separated by centrifuging or filtering out the insoluble fractions followed by inducing precipitation such as micellar precipitation or dialysis (Lam *et al.*, 2018). The extracted proteins are then dried using different methods, such as spray or freeze drying.

Yang *et al.* (2021) found that protein extraction through ultrafiltration and dialysis contained albumins, whereas alkaline extraction or micellar precipitation methods extracted mainly globulins. Not only did the different extraction methods achieve the extraction of different proteins, but the extraction method also had an impact on the protein's surface, which consequently affected the gelling properties of the extracted proteins. Alkaline extraction and salt extraction

followed by micellar precipitation or ultrafiltration achieved stronger gels, comparable to gels obtained with soy protein isolates.

After solubilization of the protein in a dilute salt solution and removal of insoluble compounds, the recovery of proteins in the supernatant solution can be achieved through micellar precipitation (MP) by diluting the solution with cold water (at 4 °C) and leaving it static to allow protein micelles to form through hydrophobic interactions. The precipitate can later be centrifuged, dialyzed, and freeze-dried (Lam *et al.*, 2018). Tanger *et al.* (2020) obtained the highest yield for AEIP, followed by SE and MP, possibly due to the loss of proteins in the solubilization and precipitation steps.

In terms of the proteins extracted, gel electrophoresis results on MP and AEIP samples showed that protein composition, while similar for both extraction methods, did not show the highest and the lowest molecular weight bands for the proteins extracted via MP. These bands, however, were present in AEIP isolates, indicating a difference in the protein composition of the different protein isolates (Paredes-Lopez *et al.*, 1991). The protein isolation method can impact the protein structure depending on the conditions the proteins are subject to during extraction (*i.e.* pH, drying method). Changes in the structure, therefore, can have an impact on the protein functionality. For example, Mwasaru *et al.* (1999) and Adebowale *et al.* (2011) reported better solubility for protein isolates extracted from pigeon pea and cowpea, as well as for Bambara groundnut, respectively, through MP when compared to those obtained through AEIP. This can be attributed mainly to the relatively harsh conditions used in the AEIP method, which led to higher protein denaturation and an observed decrease in solubility and impact on other functional properties.

2.6. Water reduction strategies in protein extraction

Alkaline extraction of proteins in pulses can be carried out using different f:w ratios. Several studies reported the use of ratios ranging from 1:5 to 1:30, where a greater difference in concentration gradients could lead to increased extractions (Lam *et al.*, 2018; Vishwanathan *et al.*, 2011). Wet extraction tends to use more water and energy than dry extraction or other combined methods, so the search for alternatives or modifications of the method is being investigated to optimize the extraction process at reduced costs (Dumoulin *et al.*, 2021). Feyzi *et al.* (2018) studied protein extraction of grass pea at different f:w ratios (from 1:5 to 1:30: v/w) in a range of pHs from 2.5 to 10 and extraction times (20-80 min). The optimal conditions for extraction were determined by measuring the extraction yield and protein contents. The authors found that extraction yield

increased with extraction time and with increasing pH (from 6 to 10). However, it was found that as yield increased, the protein content decreased, attributing this decrease to the extraction of other compounds, such as polysaccharides. Also, protein extraction increased with increasing f:w ratio at each pH condition and extraction time. However, long extraction times (higher than 50 min) showed a decreasing yield, possibly due to the denaturation of proteins and interactions of proteins and carbohydrates. The optimal conditions reported by the authors were obtained at pH 9.96, using a f:w ratio of 1:15 v/w for 58 min, obtaining the highest extraction yield. However, the highest protein content was obtained using an acidic extraction (pH 2.57) for 48 min using a 1:10 f:w ratio. Furthermore, according to the data presented by the authors, if the initial f:w ratio was decreased (*i.e.* 1:5 f:w), the protein extraction yield would not be as high as using a 1:10 f:w, but the protein content achieved would be similar.

Avila Ruiz et al. (2016) proposed a hybrid method for extracting quinoa proteins. The hybrid method proposed combines dry extraction and wet extraction using salting-in salts followed by ultrafiltration. The authors present this method as a milder option than wet extraction, allowing the proteins to be retained in their native state and reducing water usage by approximately 98%. However, the hybrid method achieves lower protein purities than conventional wet fractionation but with similar protein recovery. Overall, the proposed hybrid method is a promising approach for protein extraction, allowing for a more sustainable process.

2.7. Factors influencing protein extraction

In addition to the effects of pH, salt concentrations and temperature impacting protein extraction, the particle size of the flour feedstocks has also been shown to play a role. Russin *et al.* (2007) found a protein extraction increase of around 30% when decreasing the particle size of soy flour from 223.4 to 89.5 μm while maintaining the rest of the environmental conditions constant. Vishwanathan *et al.* (2011) found similar results while studying the particle size of soy and okara flours during protein extraction. Flours were sifted through mesh sieves (ranging from 710 to 37 μm) to separate different fractions by particle size and then subject to several consecutive step extractions. The authors found that protein recovery increased as the particle size decreased due to the increased surface area of the finer fractions.

2.8. Protein functionality

Proteins are used for their versatility as functional ingredients in various food products. Their physicochemical characteristics determine their functionality, and their amino acid composition determines their protein structure. Once incorporated into food products, proteins will interact with other components within the food system, and their behaviour will be influenced by the surrounding environment. Functional properties of proteins include solubility, emulsifying, foaming and gelling properties, water hydration and oil-holding capacities.

2.8.1. Solubility

Protein solubility is one of the most important functional properties, mainly because the interaction of proteins with water will later dictate how the proteins will perform when added to food products as emulsifiers, foaming or gelling agents (Damodaran *et al.*, 2007). Different factors can influence protein solubility, such as pH, temperature or concentration of salts. Proteins have higher solubilities at acidic or alkaline pHs where electrostatic repulsions between molecules are greater, usually far from the protein's isoelectric point. In contrast, proteins carry no net charge at the isoelectric point, and aggregation is favored due to increased attractive interactions such as van der Waals and hydrophobic forces. The presence of ions also plays a role in protein solubility, and the addition of salts can aid or hinder their solubilization, depending on the concentration and whether it can improve or disrupt the hydration layer surrounding the proteins (Shimizu *et al.*, 2006). For instance, NaCl improves solubility at low concentrations as it helps structure the hydration layer to promote protein-water interactions; however, charge screening can result in reduced solubility at higher levels. In contrast, if ammonium sulphate is added, this acts disrupting the hydration layer and exposes hydrophobic moieties on the surface. Protein solubility is reduced as proteins aggregate depending on their hydrophobicity and the ammonium sulphate concentration used. Protein solubility and temperature are directly related: an increase in the temperature will increase the protein solubility. However, if the temperature rises above the denaturation temperature, the protein’s native conformation will change, leading to protein aggregation and a decrease in solubility (Damodaran *et al.*, 2007).

Protein solubility can also be affected by processing conditions during the extraction and drying of the protein isolates. Stone *et al.* (2015b) found that the method of extraction of pea protein isolates, the cultivar, and their interaction were influential factors in the solubility of the

proteins. Among the different methods evaluated for extraction, those obtained with SE reported the highest solubilities than those obtained by AEIP or MP, attributing these results to increased hydrophobicity and protein-protein interactions occurring in the latter methods.

The drying method can also have an impact on protein solubility. Gong *et al.* (2015) studied how the drying method affected the solubility and emulsifying properties of a pea protein isolate and found that freeze-dried proteins had higher solubility than those obtained through spray drying, while Zhao *et al.* (2013) obtained opposite results for protein isolates from rice. Differences in the drying methods can lead to changes in the protein structure, exposing more hydrophobic groups to the surface of the protein, as well as denaturation, leading to aggregation that can influence the solubility of the protein isolates.

2.8.2. Emulsifying properties

An emulsion consists of two immiscible liquids, with one phase dispersed within the other. In food systems, food emulsions are mostly found as oil dispersed in water (O/W) or water dispersed in oil (W/O) systems. Emulsions are thermodynamically unfavored and, as such, require an emulsifier (*e.g.*, protein) to make them stable. Because of their amphiphilic nature, proteins adsorb in the interface of the two liquids and place the polar groups towards the water or hydrophilic phase while accommodating nonpolar groups towards the oily phase. As a result, the emulsifier lowers the interfacial tension between both immiscible liquids, stabilizing the system (Lam & Nickerson, 2013). Some factors influence the ability of proteins to perform as suitable emulsifiers, such as protein characteristics (type of protein, size, structure, concentration), preparation of the emulsion (mechanical stress, homogenization), processing conditions (temperature, pH, ionic strength, presence of other components) and time (aging) (McClements, 2004).

There are different methods available used to measure proteins' emulsifying properties. Emulsion capacity (EC) is the point at which an oil-in-water emulsion is inverted to a water-in-oil emulsion, representing the emulsified oil per gram of protein. It is measured through a drop in conductivity at the point of inversion (Can Karaca *et al.*, 2011). The emulsifying activity index (EAI) is defined by the interfacial area created in the system and expressed as area (m^2) of the interfacial area created per weight of protein (g). This method is determined using turbidimetry. The emulsifying stability index (ESI), corresponding to how stable the formed emulsion is, can also be determined using turbidimetry, measuring the change in absorbance of the same emulsion

prepared for EAI after a specific established time (Singhal *et al.*, 2016). Emulsion instability results in the separation of an oily phase and an aqueous serum layer. Therefore, the creaming stability method can be used to determine the stability of an emulsion. This is determined by measuring the serum phase separation after a certain time or over time (*e.g.,* 30 min, 1h, 2 h, 24 h) (Can Karaca *et al.*, 2011a). The availability of several methods for testing the emulsifying properties can be advantageous because it allows choosing a method that best suits the desired property to be measured; however, the availability of different non-official methods, along with varying conditions of testing used (homogenizer, speed, time) can create difficulties for comparisons of results (McClements *et al.*, 2022).

2.8.3. Foaming properties

Foams are systems of a gaseous phase dispersed in an aqueous continuous phase. Similar to emulsions, foams are also thermodynamically unstable systems, and without the help of a foaming agent, the formed foam will not be stable over time and eventually coalesce. The addition of proteins to the system and their accommodation at the interphase of the gas-aqueous phase (where hydrophilic groups will accommodate to the aqueous phase, and non-polar groups will be oriented to the gaseous phase) surrounding the gaseous bubbles formed will lower the free energy and stabilize the system to help resist against Oswald ripening (Singhal *et al.*, 2016). Foams can be prepared using different methods, the use of a homogenizer is one of the most commonly used methods in laboratory settings. However, other methods are available: physical methods such as gas sparging, chemical methods such as using substances that release CO_2 (like baking soda, for example), or biological methods such as fermentation (Drenckhan & Saint-Jalmes, 2015). Foaming properties can be determined through foaming capacity (FC), referring to the amount of foam that can be produced when a protein is added to the system, and foaming stability (FS), referring to how well the addition of a protein holds the formed foam over a specific time (Stone *et al.*, 2015b).

Some of the factors that influence FC and FS are pH (*i.e.*, near the pI protein-protein interactions are favoured, facilitating the formation of the thin film layer surrounding air bubbles), presence of salts (*i.e.*, neutralization of protein charges by the presence of ions), sugars (*i.e.*, an increase in the viscosity of the continuous phase will impede the coalescence of formed air bubbles), lipids (*i.e.*, lipids will compete with proteins to accommodate at the interphase leading

to instability) and protein concentration (*i.e.*, with increased concentration, the viscosity of the continuous phase increases, and formation of a protein thin film at the interphase is favoured) (Damodaran, 2006). Miravalles *et al.* (2019) studied the functional properties of protein isolates from lentils and found that those extracted through AE-UF showed better FC and FS than those obtained through AE-IP. These results are similar to those obtained by Boye *et al.* (2010), who found that, while there was no influence on the method of extraction in the FC of pea, chickpeas and lentil protein concentrates, there was a significant increase in FS for isolates obtained through AEIP, compared to those obtained using UF. Other authors reported similar results, where the highest FC and FS were obtained through AEIP, followed by SE and UF (Zeidanloo *et al.*, 2019), attributing this result to higher content of globulins in the AEIP isolates, therefore more exposed hydrophobic groups to the gaseous phase.

2.8.4. Water and Oil holding capacities (WHC – OHC)

Water or oil holding capacities (WHC - OHC) refer to the amount of water/oil that a certain amount of protein can hold. WHC and OHC are related to the structure of proteins, but WHC is also influenced by factors such as pH, presence of ions and temperature since these factors also affect the interactions of amino acids responsible for ionic and hydrogen bonding attractive forces (Stone *et al.*, 2015a). These properties are of great importance in the food industry because they influence the textural attributes of products such as meat or dairy products (Ma *et al.*, 2022b), baked products (Bourré *et al.*, 2019) or pastas (Bouasla *et al.*, 2017). Xu *et al.* (2017) found that WHC and OHC for chickpea isolates obtained from dry and soaked seeds showed no differences, while both properties increased if the seeds were exposed to heat, leading to a higher exposure of hydrophobic and hydrophilic groups due to unfolding of proteins, and dissociation caused by heating, allowing them to further interact with oil and water. Galves *et al.* (2019) found that defatting increased the OHC of hemp proteins due to removing lipids and allowing hydrophobic groups to interact with the oil. In contrast, the defatting process did not affect the WHC for hemp protein concentrates.

2.8.5. pH shifting as a method for protein modification

Understanding plant proteins' functional properties and performance is essential to understanding their suitability for applications. Additionally, understanding how extraction

conditions impact protein functionality is relevant for tailoring them for specific uses. However, when comparing plant proteins to animal proteins, those from animal sources generally outperform plant proteins. The main difference in their behaviour is that proteins from different origins have different structures. For example, animal proteins tend to be smaller and more flexible. In contrast, plant proteins are bigger, usually globular, and more rigid, translating to lower solubility and functionality (McClements & Grossmann, 2022). To improve the functionality of plant proteins, they can be subject to processes or modifications that can alter their structure to, for example, make them more flexible or hydrolyze them to smaller-sized proteins or peptides to partially denature or increase their hydrophobicity. These changes in structure directly impact the functionality of the proteins (Nasrabadi *et al.*, 2021).

pH shifting is a protein modification method that achieves protein changes in a simple process. Shifting the pH of the proteins to either acidic or alkaline pH allows for protein unfolding due to repulsions from charged groups of the protein, exposing previously buried hydrophobic groups towards the surface of the protein. Adjustment of the pH of the proteins back to neutrality allows protein refolding. The process of protein unfolding and refolding produces changes in the structure and surface properties, leading to increases in their solubility, emulsifying, foaming and gelling properties (Wang *et al.*, 2018). Jiang *et al.* (2012) studied both acidic (pH 1.5) and alkaline (pH 12) pH shifting as well as in combination with heat to test the film-forming abilities of a soy protein isolate and found that both pH shifting treatments achieved films that had better elasticity and stretchability, but decreased tensile strength, compared to films prepared with the native protein isolate. Figueroa-González *et al.* (2022) found that treating amaranth proteins with an alkaline pH shifting (pH 12) as well as in combination with ultrasound increased the solubility of the proteins significantly due to increased flexibility and increased interactions between proteins and water. On the contrary, the authors also examined an acidic pH shifting (pH 2) and in combination with ultrasound, and the solubility decreased significantly for these proteins, attributing increased denaturation and aggregation of the proteins at acidic pH. In conclusion, pH shifting can be a viable method for enhancing protein functionality through protein modification and facilitating the application of plant proteins into diverse products, expanding their uses.

2.9. Nutritional properties and protein quality of pulses

Proteins are composed of amino acids (AA) linked through peptide bonds. Amino acids can be classified into essential (EAA) and non-essential amino acids (NEAA). EAA (histidine, lysine, leucine, isoleucine, phenylalanine, methionine, threonine, tryptophan, and valine) are not able to be synthesized by the human body and depend only on their intake to be incorporated, while the body can synthesize NEAA. EAA intake requirements are determined based on developmental state, health conditions and special requirements such as physical state and particular circumstances of individuals (Bessada *et al.*, 2019). Pulses are high in protein (ranging from 20 to 30%) and rich in lysine, arginine, leucine, aspartic and glutamic acid. At the same time, they are low in tryptophan and sulphur-containing amino acids, cysteine and methionine. As such, they are often consumed alongside cereal as part of a complementary diet since they tend to be rich in sulphur-containing amino acids or tryptophan and deficient in lysine (Nosworthy & House, 2017).

The quality of proteins is determined by the composition of EAA and how well the body absorbs the proteins (digestibility). The digestibility of proteins is affected by different factors such as the presence of anti-nutritional factors (ANF) (*i.e.* trypsin inhibitors or phenolic compounds), food manufacturing conditions (*i.e.* modification of proteins due to the processing), protein conformation, and the presence of binding components (*i.e.* polysaccharides) (Damodaran *et al.*, 2007). Protein digestibility can be determined using *in vivo* and *in vitro* methods. *In vivo* methods consist of measuring the nitrogen present in the proteins ingested and the measurement of nitrogen content excreted (with corrections made on metabolic nitrogen). While these methods are suitable for determining true protein digestibility, they can be more costly and determinations more time-consuming than *in vitro* methods, in addition to requiring the use of animals (Nosworthy & House, 2017). *In vitro* methods to measure digestibility consist of using enzymes to simulate the digestion process, such as pepsin, pancreatin, papain, chymotrypsin or a combination of enzymes (Hsu *et al.*, 1977). However, *in vitro* methods do not consider the EAA concentration nor their availability (Bessada *et al.*, 2019). Hsu *et al.* (1977) developed a fast method for determining protein digestibility using trypsin, chymotrypsin and peptidase. A drop in pH indicated protein digestibility (pH measured after a 15 min period due to the release of AA carboxyl groups during the enzymatic digestion). This method also showed a good correlation with *in vivo* measurements.

In the US, the protein digestibility-corrected amino acid score (PDCAAS) method is used to measure protein quality. It determines not only digestibility (fecal) but incorporates the protein content and also the limiting amino acid. A result of a 100% PDCAAS shows that the protein of interest supplies sufficient amounts of all EAA. Amino acid requirements for reference proteins can be calculated according to the age group of interest, giving more accurate protein quality data for a targeted population. While this method considers both AA profile and digestibility, it can overestimate protein quality because it does not consider the presence of ANFs (such as enzyme inhibitors and phenolic compounds) (Bessada *et al.*, 2019). In Canada, the protein rating (PR) is used, which is based on the Protein Efficiency Ratio (*i.e.*, weight gained by a rat per g of ingested protein) multiplied by the grams of protein in a serving (Nosworthy & House, 2017). Nosworthy *et al.* (2017) studied the protein quality of cooked Canadian pulses (yellow and green split pea, red split lentil, green lentil, navy bean, pinto bean, black bean, red bean and kabuli chickpea). They found that all pulses had high digestibility (≥ 70%). The PDCAAS and PR values ranged from 0.67 to 0.51 and 13 – 30, respectively. With these results, the authors concluded that overall pulses could be considered protein sources according to Canada (PR values between 20 – 39.9 qualify as a source of protein) and US regulations (PDCAAS values > 0.2 qualify as a quality source of protein for non-infants).

The latest method proposed for digestibility determination is the digestible indispensable amino acid score (DIAAS). This method is similar to PDCAAS, with the main difference being that digestibility is determined on ileal digestibility instead of fecal digestibility. This difference mainly relies on the fact that fecal digestibility can be higher than ileal digestibility due to contamination from bacterial sources, impacting the digestibility by 7-22% in pigs of different growth stages (Nosworthy & House, 2017)

3. EFFECT OF PARTICLE SIZE, FLOUR: WATER RATIO AND TYPE OF PULSE ON THE PHYSICOCHEMICAL AND FUNCTIONAL PROPERTIES OF WET PROTEIN EXTRACTION[1]

3.1. Abstract

Extraction of proteins by alkaline extraction followed by isoelectric precipitation at three different flour: water (f:w) ratios (1:10, 1:7, and 1:5) were investigated for four pulse flours: yellow pea (YP), green lentil (GL), kabuli chickpea (CH), and navy bean (NB). Pulse flours were prepared by pre-breaking the seeds and milling into flour using a Ferkar (knife) mill with either a 0.5 mm (P1) or 1.27 mm (P2) screen. Surface properties and functionality of the protein concentrates were evaluated. Extraction yields showed no major differences between the investigated f:w ratios. Protein yields decreased significantly as the amount of water decreased. The 1:10 f:w ratio was selected to perform extractions with coarser flours (1.27 mm; P2). Results showed that flour with a smaller particle size increased yields by 0.4% to 3.2% for extraction, and 7.9%–10.3% for protein. Functional properties showed no major differences between proteins extracted at different f:w ratios, although differences were found between the different pulse types. In some cases, P2 and P1 concentrates differed in functional properties, but this was not consistent for all pulses. In summary, finer flour (P1) and higher f:w ratio (1:10) resulted in higher extraction and protein yields. This study highlights the importance of milling and control of the particle size of the flours and water use on the preparation of protein concentrates.

Introduction

Pulses are extensively consumed around the world and widely grown across Canada for their nutritional profile and health benefits, as well as for their uses in the food industry (Boye *et al.*, 2010; Stone *et al.*, 2015a). The growing interest for alternative protein sources has increased the

[1] Higa, F. A., Boyd, L., Sopiwnyk, E., & Nickerson, M. T. (2022). Effect of particle size, flour:water ratio and type of pulse on the physicochemical and functional properties of wet protein extraction. Cereal Chemistry, 99(5), 1049–1062.https://doi.org/10.1002/cche.10

attention on these crops due to their high protein content (~21%–25%) and added value through protein fractionation and extraction (Rochfort & Panozzo, 2007; Singh, 2017). Proteins in pulses are mainly comprised of storage proteins; salt soluble globulins comprising 35%–72% of the total protein content, while the rest is comprised by water-soluble albumins (Singh, 2017). Legumins (11*S* hexameric, 300–400 kDa) are constituted of acidic (α: 40 kDa) and basic (β: 20 kDa) subunits linked through disulfide bonds, while vicilins (7*S* trimeric, glycosylated proteins, 150–190 kDa) are comprised of 50–70 kDa monomers that are linked through noncovalent bonds (Shevkani *et al.*, 2019). The monomers are formed by α, β, and γ subunits that can result in 30–37 kDa fragments (Lam *et al.*, 2017). Convicilin is also a 7*S* storage protein with a molecular weight of ~70 kDa, and unlike vicilins, convicilin possesses sulfur containing amino acids (Croy *et al.*, 1980). Legumin and vicilin ratios increase during the development of seeds due to the synthesis of the different proteins. These ratios are affected by factors like environmental conditions, cultivar (Lam *et al.*, 2018), and pulse type (Guldiken *et al.*, 2021; Martinez *et al.*, 2016; Tavano & Neves, 2008).

Protein extraction can be achieved by dry or wet fractionation methods. The latter method achieves higher protein content fractions than those obtained through air classification (Singhal *et al.*, 2016). Protein separation can be achieved using different wet extraction methods (*i.e.*, alkaline extraction, salt extraction, micellar precipitation) and extraction can be optimized through the modification of parameters such as solute to solvent ratio, pH, temperature, and time (Feyzi *et al.*, 2018; Can Karaca *et al.*, 2011a; Kiosseoglou & Paraskevopoulou, 2011). Alkaline extraction followed by isoelectric precipitation (AEIP) involves solubilizing the proteins at alkaline pH and precipitation at the protein's isoelectric point, where the protein will have no net charge and protein–protein interactions will be favored. This method has been widely used and reported in previous research (Ghribi *et al.*, 2015; Kaur & Singh, 2007; Lam *et al.*, 2017; Papalamprou *et al.*, 2010). For example, Gao *et al.* (2020) found that increasing pH resulted in higher extraction yields of yellow pea (YP). However, the isolated proteins tended to aggregate, and therefore had reduced solubility. Du *et al.* (2018) reported no significant increases in protein yields when the temperature increased from 30 to 40°C and then to 50°C when extracting mung bean protein. However, other authors reported that an increase in temperature favored the extraction yield but resulted in protein denaturation followed by a decrease in functional properties and nutritional value due to the loss of lysine, cysteine, serine, and threonine (Lam *et al.*, 2018; Swanson, 1990; Zhang *et al.*, 2018).

Protein isolation through wet extraction methods requires high amounts of water to be used compared to dry fractionation (Avila Ruiz *et al.*, 2016). For example, the extraction of a lupin protein isolate requires approximately ~87 kg water/kg protein, as well as high energy requirements for the final drying step which in turn results in higher costs. A reduction of water in the extraction process can also lead to a decreased environmental impact and a more sustainable process (Berghout *et al.*, 2015). The present study investigated the effect of flour:water ratios (commonly used 1:10 flour to water ratio, and a reduction of water to 1:7 and 1:5 ratios) on AEIP extracted proteins, as well as the particle size of the pulse flours on the protein extraction yields of different pulses (yellow pea, green lentil, chickpea, and navy bean). These pulses are widely grown in Canada and were selected for this study to better understand how different parameters can affect protein extraction in different pulses. Protein functionality (*e.g.*, solubility, water/oil holding, emulsification, and foaming) is strongly related to the physicochemical properties of the proteins, for instance, their charge and hydrophobicity, their protein profile, and their composition. Knowledge of structure– function relationships within different food environments is essential to tailor the ingredients to certain applications. Researching water reduction strategies is also important for industry as water consumption and drying in wet protein fractionation plants is costly.

3.2. Materials and Methods

3.2.1. Materials

Green lentil (GL) (Crop development center [CDC] Greenstar), kabuli chickpea (CH) (CDC Orion), and YP (CDC Spectrum) were harvested in the 2018 crop year and were obtained from Reisner Farm Ltd., whereas navy bean (NB) (Nautica) was purchased from Hensall Co-op. Flours were prepared by pre-breaking the seeds using a hammer mill (Jacobson Model 120-B; Jacobson Machine Works Inc.) fitted with an 8/64″ screen, followed by milling with a Ferkar multipurpose knife mill (Ferkar 5 Model; KFM, d.o.o.) using either a 0.5 mm (particle size 1; P1/fine) or 1.27 mm (particle size 2; P2/coarse) screen in duplicate.

3.2.2. Particle size distribution

Particle size of the flours was determined by laser diffraction with dry dispersion using the Malvern Mastersizer 2000 with Scirocco 2000 accessory (Malvern Instruments Inc.). The mean

values of uniformity (as the measure of the absolute deviation from the median particle size), D[4,3] (volume moment mean, reflecting the size of the bulk of the sample volume), and percentiles; d10, d50, and d90 (reflecting the maximum particle size for 10%, 50%, and 90% of the sample volume) were measured.

3.2.3. Preparation of pulse protein concentrates

Before extraction, flour samples were defatted using hexane (1:3 w/v) for 40min following the procedure described by Stone *et al.* (2015a). A total of three subsequent defatting steps were done filtering the sample through Whatman #1 paper (Whatman International Ltd.), followed by drying of the sample in a fumehood overnight. AEIP protein extraction was performed in triplicate following the procedure described by Can Karaca *et al.* (2011a). P1 flours were dispersed at 1:10, 1:7, and 1:5 flour:water (f:w) (w/v) ratios using Milli-Q water, then pH adjusted to 9.5 using 2M NaOH with agitation (400 rpm) for 1 h. Samples were then centrifuged at 4500g for 15min at 4°C using a Sorvall RC-6Plus centrifuge (Thermo Scientific) to separate the solubilized proteins in the supernatant from insoluble constituents. The pH of the supernatant was then adjusted to 4.5 with 1M HCl to precipitate the proteins, followed by centrifugation at 4500g for 15min at 4°C, and were then stored at −30°C until freeze-dried (Labconco FreeZone 6 freeze dryer; Labconco). Protein extraction from P2 flours were prepared using a 1:10 f:w ratio (v/w) only, following the same extraction procedure described previously.

3.2.4. Protein content

Protein content in the flours and protein concentrates were determined using a LECO FP 628 series (LECO Corp., St. Joseph, MI, United States) in duplicate determination following the combustion method (992.15; AOAC International, 1995). A protein conversion factor of 6.25, commonly used for pulses, was used.

3.2.5. Protein and extraction yields

Yields were calculated as follows:

$$\text{Extraction yield} = \frac{\text{weight of protein concentrate (g)}}{\text{weight of defatted flour (g)}} \text{ x } 100 \qquad (3.1)$$

$$\text{Protein yield} = \frac{protein\ content\ in\ concentrate\ (g)}{protein\ content\ in\ the\ flour\ (g)} \text{ x } 100 \quad (3.2)$$

The f:w ratio used in the protein extraction of P1 flours that resulted in highest yields was selected for the protein extractions of the P2 flours.

3.2.6. Physicochemical properties

3.2.6.1. Surface charge

Zeta potential was determined as described by Stone *et al.* (2015a) using a Zetasizer Nano ZS90 (Malvern Instruments). Electrophoretic mobility was determined from prepared protein solutions (0.05%w/w) where pH was adjusted to 7.0 using 0.05M NaOH or 0.05M HCl and stirred at 4°C overnight. Zeta potential (ζ) was calculated using Henry's Equation (3.3):

$$U_E = \frac{2\varepsilon\,\zeta\,f\,(\kappa\alpha)}{3\eta} \quad (3.3)$$

where ε is the permittivity of the medium (Farad/m), f(κα) is a function of the particle radius (α) and Debye length (κ), and η represents viscosity (mPa·s). Measurements were determined in duplicate.

3.2.6.2. Surface hydrophobicity

Surface hydrophobicity was determined with the 8-anilino-1-naphtalensulfonic acid (ANS) probe, following the method of Kato & Nakai (1980) with some modifications. Protein solutions were prepared at 0.025% w/w with Milli-Q water, and the pH was adjusted and maintained for 1 h while stirred. Dilutions were prepared (0.005%, 0.010%, 0.015%, and 0.020% w/w) followed by the addition of 20 µl of ANS and vortexed for 10 s and kept for 5min in the dark. Samples were measured with an excitation and emission wavelengths of 390 and 470 nm, respectively, using a spectrofluorometer (Fluoromax-4; Horiba Jobin Yvon Inc., Edison, NJ, USA). Blanks for the protein and ANS were subtracted from the measurements. The resulting slope of protein fluorescence versus protein concentration was determined using linear regression and represented the protein surface hydrophobicity (H_0).

3.2.6.3. Legumin/Vicilin ratio

Sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE) was performed under reducing conditions following the method by Laemmli (1970) to quantify the legumin and vicilin ratios of the protein concentrates. A 15% separating gel was used at pH 8.6, and a 4% stacking gel at 6.8 pH. Protein solutions were prepared (1% w/w) and stirred overnight. Sample preparation for gel loading consisted of 30 µL of sample and 30 µl of Laemmli buffer (20 mM Tris-HCl buffer at pH 7.6, 50% glycerol, 10% SDS solution, 0.01% bromophenol blue, and 2% β-mercaptoethanol) vortexed for 10 s and heated at 85°C in a water bath for 10 min. The samples were then centrifuged at 12,100g for 10 min and loaded into the prepared gel. The samples were run in a MGV-202 Vertical mini-gel system (CBS Scientific) for 1.5 h at 120 V and 40 mPa with a 300 V power source (VWR). A molecular weight marker ranging from 5 to 245 kDa (BLUelf Prestained Protein Ladder; FroggaBio Scientific Solutions) was used. The gel was fixed overnight with a gel fixing solution prepared with methanol, glacial acetic acid, and water (5:1:4, v-v:v) and later stained for 1 h with a 0.1% Coomassie blue stain, followed by destaining with a methanol– water–glacial acetic acid (3:6:1, v-v:v) solution for 4 h. The obtained protein bands were imaged using ImageJ (National Institutes of Health) and their molecular weights estimated against the standards. The protein bands were determined by pixel intensity. Measurements were determined in duplicate.

3.2.7. Functional properties

3.2.7.1. Protein Solubility

Protein solubility was determined using the method described by Lam *et al.* (2017). The nitrogen content present in the supernatant was determined in protein solutions prepared at 1% (w/w) based on the protein content present in the concentrate. The solutions pH were adjusted and maintained at pH 7.0 using 0.5M NaOH or HCl and stirred at 500 rpm for 1 h at room temperature. Solutions were then centrifuged at 4180g for 10 min at room temperature. The protein content of the supernatant and concentrates was determined using a micro-Kjeldahl digestion unit (Labconco Co.), using a correction factor of 6.25. Protein solubility (%) was determined as the protein content in the supernatant divided by the protein content in the concentrate sample.

3.2.7.2. Emulsion activity index (EAI) and emulsion stability index (ESI)

EAI and ESI were determined as described by Avramenko *et al.* (2013) with modifications. Protein solutions (0.25% w/w) were prepared by dispersing protein concentrates in water and stirred for 1 h (400 rpm) while pH was adjusted and maintained at 7.0. Emulsions were prepared on an IKA homogenizer (T-10 Basic ULTRA-TURRAX; IKA Works, Wilmington, NC, USA) fitted with an S-10 N - 10 G dispersing tool, homogenizing the protein solutions with 4.0 g of canola oil at speed 5 (12,800 rpm) for 5 min. Aliquots of the emulsion (50 µL) were taken at times 0 and 10 min and were diluted with 7.5 ml of sodium phosphate buffer (pH 7.0, 10 mM) with 0.1% SDS and vortexed for 10 s. Absorbance measurements were performed at 500 nm (Genesys 10; Thermo Scientific) using 1 cm length plastic cuvettes. EAI and ESI were calculated as follows:

$$EAI\ (\frac{m^2}{g}) = \frac{2 \times 2.203 \times A_0 \times N}{c \times \varphi \times 10000} \quad (3.4)$$

$$ESI\ (\text{min}) = \frac{A_0}{\Delta A} \times t \quad (3.5)$$

where A_0 corresponds to the absorbance of the system measured after homogenization, F is the dilution factor, c corresponds to the protein solution expressed in g/ml and φ represents the oil volume fraction in the emulsion, A_0 corresponds to the initial absorbance of the emulsion, and ΔA represents the change in absorbance between 0 and a fixed amount of time (t = 10 min).

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

FC and FS were determined using an IKA homogenizer (T-10 Basic ULTRA-TURRAX; IKA Works, Wilmington, NC, USA) fitted with an S-10 N - 10 G dispersing tool as described by Lam *et al.* (2017) and Stone *et al.* (2015b). Protein solutions were prepared at 1.0% (w/w) in water, pH adjusted and maintained at pH 7.0 while stirred for 1 h at room temperature. The protein solution (15 ml) was homogenized in a 400 ml glass beaker for 5 min at speed 5 (12,800 rpm). After homogenization, the foam was transferred to a 100 ml graduated cylinder. At times 0 and 30min the volume of foam was recorded (ml), and FC and FS were calculated as shown in Equations (3.6) and (3.7):

$$\%FC = \frac{V_0}{V_i} \times 100 \quad (3.6)$$

$$\%FS = \frac{V_f}{V_0} \times 100 \quad (3.7)$$

where V_0 is the foam volume at time 0 (ml), V_i refers to the initial volume of the sample (15 ml), and V_f is the volume measured at time 30min.

3.2.7.4. Water hydration capacity (WHC) and oil holding capacity (OHC)

WHC and OHC were determined following the procedure described by Stone *et al.* (2015a). Pre-weighed 30 ml centrifuge tubes were used to weigh 0.25 g of protein and 5 g of water or oil into. The sample was vortexed for 10 s every 5 min for a total of 30 min (S/P® Vortex Mixer; Baxter Diagnostics Inc.). The samples were later centrifuged at 1000g for 15min (Sorvall® SS-1 Superspeed Angle Centrifuge; Thermo Scientific) and the supernatant discarded. WHC and OHC were calculated by dividing the gained weight of the sample by its initial weight.

3.2.8. Statistical Analysis

Extractions were performed in triplicate and reported as the mean ± 1 standard deviation. Physicochemical and functional properties were performed in duplicate for each of the triplicate extractions (n = 6). Statistical analyses were performed using RStudio Version 1.2.5019 (RStudio, Inc.). One-way analysis of variance with Scheffe test was performed to determine differences between means for the extraction and protein yields, as well as the physicochemical and functional properties.

3.3. Results and discussion

3.3.1. Particle size distribution

The flour particle size distribution results are shown in Table 3.1. The difference between the particle sizes of P1 and P2 flours for each pulse differed by more than 200 µm for D[4,3]. For instance, YP P1 and P2 produced flours with D[4,3] of 85.7 and 413.0 µm, respectively; GL had values of 120.0 and 368.3 µm, respectively; CH had values of 62.8 and 312.3 µm, respectively; and NB had values of 141.9 and 369.3 µm, respectively. Furthermore, flours produced using the

1.27 mm screen (P2) produced particle size distributions with greater uniformity, whereas those produced using the 0.5 mm screen (P1) had distributions which were more polydispersed.

Table 3.1. Particle size analysis of pulse flours and their protein contents (% d.b.). Data represent the mean ± one standard deviation.

Description	d(0.1) (μm)	d(0.5) (μm)	d(0.9) (μm)	D[4,3] (μm)	Uniformity	Protein content
YP P1	8.07 ± 0.14	32.08 ± 0.12	249.37 ± 0.66	85.69 ± 0.19	2.15 ± 0.02	24.17 ± 0.04
YP P2	18.28 ± 0.04	340.37 ± 0.02	944.91 ± 16.04	412.96 ± 4.66	0.87 ± 0.01	24.08 ± 0.08
GL P1	11.21 ± 0.04	89.11 ± 0.12	284.09 ± 0.04	119.96 ± 0.04	1.02 ± 0.00	24.42 ± 0.02
GL P2	24.46 ± 0.20	332.28 ± 3.89	752.13 ± 12.37	368.27 ± 5.35	0.66 ± 0.00	25.24 ± 0.01
CH P1	8.53 ± 0.05	23.23 ± 0.00	194.63 ± 0.06	62.84 ± 0.07	2.12 ± 0.00	20.16 ± 0.08
CH P2	15.97 ± 0.08	229.57 ± 0.60	769.54 ± 10.71	312.33 ± 3.42	1.08 ± 0.02	19.93 ± 0.05
NB P1	7.16 ± 0.04	37.06 ± 0.51	317.95 ± 2.73	141.88 ± 0.53	3.36 ± 0.04	25.64 ± 0.02
NB P2	17.17 ± 0.06	302.03 ± 0.67	847.70 ± 13.06	369.35 ± 4.33	0.87 ± 0.01	25.70 ± 0.09

Abbreviations: YP, yellow pea; GL, green lentil; CH, chickpea; NB, navy bean. P1- Screen size 0.5 mm, P2- Screen size 1.27 mm

3.3.2. Protein contents, extraction, and protein yields

The protein contents of the flours and extracted protein concentrates are shown in Tables 3.1 and 3.2. The obtained values for the flours were within the range of previous research for pulse flours (Bourré *et al.*, 2019; Guldiken *et al.*, 2021; Sánchez-Vioque *et al.*, 1999) as well as for extracted proteins using the same AEIP extraction method (Can Karaca *et al.*, 2011b; Jarpa-Parra, 2018; Sánchez-Vioque *et al.*, 1999). Protein contents in the flours were approximately 24% for YP, 25% for GL, 20% for CH, and 25% for NB and were similar between the different particle-sized flours (P1 and P2). The protein content of the concentrates ranged from ~88% to 93% for YP, ~84% to 88% for GL, ~84% to 90% for CH, and ~73% to 81% for NB.

The extraction and protein yields are presented in Table 3.2. The extraction yields (referred to the amount of protein concentrate obtained from the flour) ranged from 14.8% to 19.2% and were comparable with those found in literature for pulses (Stone *et al.*, 2015b). A decrease in extraction yield was observed with decreasing f:w ratio used. However, these differences were not significant, except for YP, where the 1:5 ratio was significantly different from 1:7 to 1:10 extraction ratios.

Feyzi *et al.* (2018) found similar results when studying the optimal extraction conditions for grass pea protein, where increasing water to flour ratios resulted in an increase in extraction yields.

However, the f:w ratio had a significant effect on the protein yields obtained (protein recovered in the protein concentrate as a proportion of the protein content in the flour) (Table 3.2). As the amount of water used in the extraction decreased, the protein yield of the concentrates decreased significantly for all pulse types.

The protein yields difference ranged between 4.1% and 7.5% for the different pulse types between the 1:10 and the 1:5 f:w ratios used in protein extraction. These results follow mass transfer principles, where the driving force for extraction is given by the difference in the concentration gradient between the liquid (water) and the solid (pulse flours), and therefore are higher when the amount of solvent (water) used is greater (Pinelo *et al.*, 2005). The protein yield of the concentrates ranged from ~50.9% to 69.8% (Table 3.2). Lam *et al.* (2017) and Soetrisno & Holmes (1992) reported similar protein yields for the AEIP proteins for pea, chickpeas, and lentils extracted at 1:10 f:w ratios.

Based on the results, the f:w ratio of 1:10 was selected for further extractions with the coarser particle size (P2). Results showed an effect of particle size on the yields, where flours with a smaller particle size had higher extraction and protein yields for all protein concentrates. The increase in the extraction and protein yields ranged from 0.4%–3.2% to 7.8%–12.2%, respectively. In line with the obtained results, Vishwanathan *et al.* (2011) found a 92% protein recovery in the first extraction for the coarser fraction of soybean flour, while the fine fraction yielded a 3% higher protein recovery. However, the authors reported no changes in protein recovery for different particle sizes of okara flour. Russin *et al.* (2007) reported an increase in yields when extracting proteins from soy flour of different particle sizes: 89.5 ± 1.1 mm, 184.2 ± 1.6 mm, and 223.4 ± 6.4 mm. The authors found an increase of more than 32% and 30% between the smallest and largest particle sizes for extraction and protein yields, respectively, prepared using AEIP at pH 9.0 using a 1:15 f:w ratio. In the present study, CH presented the lowest extraction yields for P1 and P2 flours, along with YP P2 and NB P2. However, the protein yields in the CH concentrates were the highest along with GL, followed by YP, and then NB with the lowest protein yield of all pulses. Differences in the obtained yields among different pulses can be attributed to their initial composition, where the presence of soluble fiber can act as an interference during protein extraction.

Table 3.2. Extraction and protein yields (%, d.b.) of AEIP protein concentrates using different f:w ratios, followed by isoelectric precipitation from different particle sized flours and pulses. Data represents the mean ± one standard deviation (n=3).

Pulse/Size	Extraction yields (% d.b.)		
	1:10	**1:7**	**1:5**
YP P1	16.98 ± 0.25 ^{Aa}	16.84 ± 0.16 ^{a}	16.00 ± 0.18 ^{b}
YP P2	14.77 ± 0.09 ^{B}	N/A	N/A
GL P1	19.08 ± 0.06 ^{Aa}	18.84 ± 0.07 ^{a}	18.88 ± 0.15 ^{a}
GL P2	17.09 ± 0.24 ^{B}	N/A	N/A
CH P1	15.54 ± 0.18 ^{Aa}	15.50 ± 0.20 ^{a}	15.49 ± 0.23 ^{a}
CH P2	15.11 ± 0.08 ^{B}	N/A	N/A
NB P1	19.12 ± 0.47 ^{Aa}	19.22 ± 0.40 ^{a}	18.59 ± 0.20 ^{a}
NB P2	15.93 ± 0.08 ^{B}	N/A	N/A
Pulse/Size	**Protein yields (% d.b.)**		
	1:10	**1:7**	**1:5**
YP P1	65.24 ± 0.51 ^{Aa}	63.17 ± 0.39 ^{b}	58.75 ± 0.35 ^{c}
YP P2	56.14 ± 0.30 ^{B}	N/A	N/A
GL P1	69.76 ± 0.26 ^{Aa}	67.29 ± 0.33 ^{b}	65.60 ± 0.60 ^{c}
GL P2	57.52 ± 0.48 ^{B}	N/A	N/A
CH P1	69.41 ± 0.46 ^{Aa}	67.67± 0.85 ^{a}	64.98 ± 0.96 ^{b}
CH P2	61.58 ± 0.35 ^{B}	N/A	N/A
NB P1	61.21 ± 1.42 ^{Aa}	58.00 ± 1.19 ^{b}	53.74 ± 0.36 ^{c}
NB P2	50.87 ± 0.32 ^{B}	N/A	N/A
Pulse/Size	**Protein content (% d.b.)**		
	1:10	**1:7**	**1:5**
YP P1	92.75 ± 0.62 ^{Aa}	90.59 ± 0.38 ^{b}	88.64 ± 0.65 ^{c}
YP P2	90.79 ± 0.19 ^{B}	N/A	N/A
GL P1	88.53 ± 0.24 ^{Aa}	86.49 ± 0.24 ^{b}	84.14 ± 0.16 ^{c}
GL P2	84.28 ± 1.48 ^{B}	N/A	N/A
CH P1	90.13 ± 0.48 ^{Aa}	88.08 ± 0.52 ^{b}	84.65 ± 0.55 ^{c}
CH P2	84.93 ± 0.51 ^{B}	N/A	N/A
NB P1	81.37 ± 0.54 ^{Aa}	76.72 ± 0.78 ^{b}	73.51 ± 0.32 ^{c}
NB P2	81.54 ± 0.16 ^{A}	N/A	N/A

Abbreviations: YP, yellow pea; GL, green lentil; CH, chickpea; NB, navy bean. P1- Screen size 0.5 mm, P2- Screen size 1.27 mm. - *Note:* NA (not applicable) - Lowercase letters show significant differences within pulse type for different f:w ratios ($p < 0.05$). Uppercase letter shows significant differences between different particle sizes of the same pulse ($p < 0.05$).

The results showed that a reduction in the water used for extraction leads to a decrease in the extraction and protein yields. However, reducing the particle size of the pulse flours, using the same f:w ratio, can lead to an increase in the amount of protein extracted. Therefore, controlling the particle size of the flour before extraction could be a useful method for obtaining higher protein yields with less use of water.

3.3.3. Physicochemical properties

3.3.3.1. Surface properties

The surface charge for all concentrates showed a net negative charge, with zeta potential values that ranged from −42.72 to −32.10 mV at pH 7.0 (Table 3.3). Can Karaca *et al.* (2011b) reported higher zeta potential values and found that legume source as well as the extraction method and their interaction, had an effect on the surface charge, reporting values ranging from −23 to −21 mV for chickpeas and lentils extracted by AEIP at 1:10 f:w ratios. Surface charge of proteins, as a result of ionizations of groups on the surface of the proteins and disassociations of globulins to their subunits during extraction (Gueguen *et al.*, 1988), can explain the differences in zeta potential of the different pulses. Results showed that f:w ratio used had an effect on the zeta potentials of the protein concentrates, where the lowest zeta potentials obtained corresponded to those concentrates extracted at 1:10 f:w ratio, followed by extraction at 1:7 and 1:5 ratios. For all f:w ratios tested, the lowest zeta potential was seen for CH, followed by YP, GL, and NB. Shevkani *et al.* (2015) reported that kidney beans had lower zeta potential values than those found for field pea protein isolates. Proteins extracted from the P2 flours showed a significant increase in zeta potential compared to those obtained from P1 flours. This could be due to extractions resulting from smaller particle-sized flours (which were also found to be more polydispersed than the coarser flours, showing a wider range of finer particles) resulting in more efficient extraction of globulins due to increased surface area followed by disassociation of subunits at alkaline pH, resulting in lower zeta potential values. These results suggest that the electrostatic repulsive forces between proteins are high, and water-protein interactions are favored in the tested conditions at pH 7.0.

Surface hydrophobicity values obtained ranged from ~14 to 149 a.u. and are shown in Table 3.3. Stone *et al.* (2015a) and Stone *et al.* (2015b) reported similar values for pea protein isolates prepared using AEIP (22.3–25.9 a.u.), while Can Karaca *et al.* (2011b) showed surface hydrophobicity values for chickpeas and faba beans in the range of those obtained in the present

study. No major differences were found in hydrophobicity for the concentrates extracted at different f:w ratios. Proteins extracted from the P2 flours showed higher hydrophobicity than those obtained from P1, except for NB, where lower hydrophobicity for P2 was also accompanied by an increase in solubility. Some hydrophobicity results will be discussed in the following section on functionality testing. Hydrophobicity results, along with solubility can contribute to the understanding of functionality, given that in order for the proteins to decrease the interfacial tension in emulsions/foams, they need to solubilize to be able to migrate to the interface, and to accommodate their hydrophobic moieties on the surface of the protein towards the oil/air interface.

Table 3.3. Zeta potential, surface hydrophobicity and legumin/vicilin ratios of protein concentrates extracted at different f:w ratios for different pulses and flours of particle sizes P1 and P2. Data represents the mean ± one standard deviation (n=6).

Pulse/Size	Zeta potential (mV)		
	1:10	**1:7**	**1:5**
YP P1	-42.72 ± 1.40 ^{Aa}	-40.18 ± 1.33 ^{b}	-40.91 ± 1.88 ^{b}
YP P2	-36.08 ± 1.15 ^{B}	N/A	N/A
GL P1	-38.52 ± 1.22 ^{Aa}	-38.03 ± 1.40 ^{a}	-36.38 ± 1.75 ^{b}
GL P2	-35.95 ± 1.48 ^{B}	N/A	N/A
CH P1	-43.06 ± 1.94 ^{Aa}	-42.46 ± 1.43 ^{ab}	-41.56 ± 1.66 ^{b}
CH P2	-40.56 ± 1.37 ^{B}	N/A	N/A
NB P1	-35.96 ± 2.46 ^{Aa}	-32.88 ± 0.59 ^{b}	-32.10 ± 0.94 ^{b}
NB P2	-36.74 ± 1.12 ^{A}	N/A	N/A
Pulse/Size	**Surface Hydrophobicity (a.u.)**		
	1:10	**1:7**	**1:5**
YP P1	29.00 ± 12.84 ^{Ba}	38.03 ± 13.76 ^{a}	29.81 ± 9.13 ^{a}
YP P2	149.05 ± 14.13 ^{A}	N/A	N/A
GL P1	14.19 ± 2.59 ^{Ba}	10.92 ± 3.34 ^{a}	14.27 ± 7.67 ^{a}
GL P2	99.70 ± 10.33 ^{A}	N/A	N/A
CH P1	28.74 ± 11.49 ^{Aa}	21.05± 8.75 ^{a}	19.73 ± 4.69 ^{a}
CH P2	47.12 ± 20.14 ^{A}	N/A	N/A
NB P1	84.32 ± 17.84 ^{Ab}	121.49 ± 13.53 ^{a}	120.98 ± 9.57 ^{ab}
NB P2	52.04 ±16.91 ^{B}	N/A	N/A
Pulse/Size	**L/V ratios**		
	1:10	**1:7**	**1:5**
YP P1	0.96 ± 0.25 ^{Aa}	0.88 ± 0.18 ^{a}	0.85 ± 0.22 ^{Aa}
YP P2	0.95 ± 0.24 ^{A}	N/A	N/A
GL P1	0.69 ± 0.08 ^{Aa}	0.69 ± 0.07 ^{a}	0.63 ± 0.08 ^{a}
GL P2	0.63 ± 0.12 ^{A}	N/A	N/A
CH P1	1.88 ± 0.19 ^{Aa}	1.97 ± 0.21 ^{a}	2.05 ± 0.21 ^{a}
CH P2	2.22 ± 0.14 ^{B}	N/A	N/A
NB P1	0.10 ± 0.04 ^{Ab}	0.12 ± 0.02 ^{ab}	0.15 ± 0.02 ^{Aa}
NB P2	0.09 ± 0.02 ^{A}	N/A	N/A

Abbreviations: YP, yellow pea; GL, green lentil; CH, chickpea; NB, navy bean. P1- Screen size 0.5 mm, P2-Screen size 1.27 mm. *Note:* NA (not applicable). Lowercase letters show significant differences within pulse type for different f:w ratios ($p < 0.05$). Uppercase letter shows significant differences between different particle sizes of the same pulse ($p < 0.05$).

3.3.3.2. Legumin and vicilin protein ratios

Determination of legumin and vicilin was performed through an electrophoretic technique (SDS-PAGE). Observed bands ranged from ~10 to 82 kDa. Under the reducing conditions of SDS-PAGE, vicilin was found in its dissociated subunits of ~33−35 kDa (α and β) and 47−50 kDa (α, β, and γ), while legumin was observed in its acidic (α: 40kDa) and basic (β: 20 kDa) subunits. The observed band of ~70 kDa was presumed to correspond to convicilin (Gao *et al.*, 2020; Ladjal-Ettoumi *et al.*, 2016; Rui *et al.*, 2011; Xu *et al.*, 2017). An example of SDS-PAGE bands for each pulse are presented in Figure 3.1. The legumin/ vicilin (L/V) ratios found are shown in Table 3.3. The mean L/V ratios were highest for CH followed by YP, GL, and NB. The ratios obtained were similar, or in the range, of those reported by other authors (Guldiken *et al.*, 2021; Lam *et al.*, 2017; Swanson, 1990; Tavano & Neves, 2008). For example, Tavano & Neves (2008) reported a L/V ratio of ~3.6 for CH while the values obtained in our research ranged from 1.88 to 2.22. It has been reported that growing conditions and environmental variations (rainfall, temperature, etc.) can influence the L/V ratios in pea (Bourgeois *et al.*, 2009). Cultivar and maturity stage at harvest can also have an impact on L/V ratio (Lam *et al.*, 2018). No differences were found in the L/V ratios between the different f:w ratios used, except for NB where the L/V ratio for the 1:10 extracted concentrate was significantly lower than the concentrate obtained at 1:5 f:w ratio. These results are in agreement with the protein solubility results (Table 3.4), where the solubility was significantly higher for the 1:10 f:w ratio. This could be explained due to the higher amounts of vicilin, which has been reported to have more glutamic and aspartic acid than legumin, and consequently favored protein-water interactions (Guldiken *et al.*, 2021; Lam, 2016). NB also had the highest WHC of the pulses investigated.

The L/V ratios of concentrates obtained from different particle-sized flours showed no significant differences except for CH, where the L/V ratio was higher for P2 than P1. In line with these results, surface hydrophobicity results showed that CH P2 had higher H_0 than P1, possibly due to higher legumin content in P2, presenting more surface area than vicilin, exposing tryptophan residues and resulting in higher surface hydrophobicity (Koyoro & Powers, 1987).

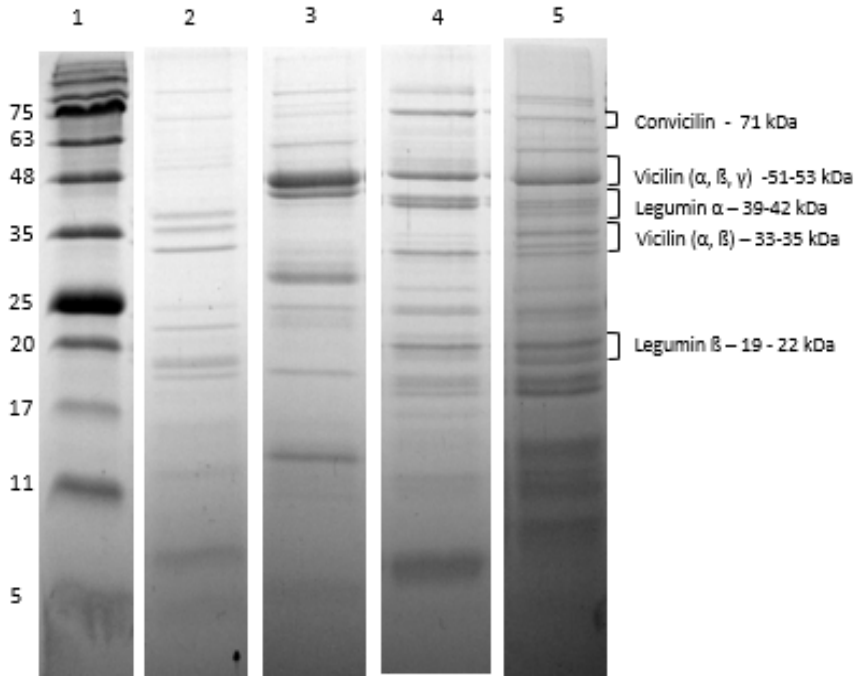

Figure 3.1. Example of SDS-PAGE on protein concentrates extracted with P2 flour (1.27 mm screen size). Lanes: (1) molecular weight marker, (2) P2 chickpea, (3) P2 navy bean, (4) P2 yellow pea, (5) P2 green lentil.

3.3.4. Functionality testing

3.3.4.1. Protein solubility

Protein solubility is important since other functional properties (*i.e.*, surface properties) are related to the solubilization of the proteins (Shevkani *et al.*, 2019). It is also important for the application of isolates or concentrates to other products. Solubility is influenced by both intrinsic (amino-acid composition, L/V ratios, charge) and extrinsic factors (pH, temperature). As extrinsic factors were kept constant any differences in solubilities can be attributed to differences in the protein composition, the distribution of hydrophobic and hydrophilic groups present in the protein surface and the interaction with other proteins (Damodaran *et al.* 2007). Zeta potential values for all extracted samples showed negative values suggesting favourable protein-water interaction, therefore high solubilities. Solubility values ranged from ~53% to ~87% for the pulses examined (Table 3.4). The highest solubility was observed for NB concentrates prepared with both particle-sized flours (P1 and P2). The SDS-PAGE analysis revealed the lowest L/V ratios for NB. The presence of higher amounts of vicilin, which has been reported to have more glutamic and aspartic

acid than legumin, could explain the high solubility results (Lam, 2016). The GL concentrate from P1 flour had the lowest solubility for all pulses (~53%–60%). Similar solubility values determined at pH 7 for red lentil have been reported in the literature when the same extraction method was used (~55%; Jarpa-Parra *et al.* 2015). Solubility values for yellow pea were similar to the range reported by Stone *et al.* (2015b) for different pea cultivars (~58%–76%), as well as for desi chickpea (~55%) reported by Boye *et al.* (2010)

Differences were found in solubilities from proteins extracted from GL where P2 concentrates showed higher solubilities than P1. This could be attributed to possible aggregation of the proteins from P1 concentrates, exposing less hydrophobic residues, resulting in soluble aggregates that presented lower surface hydrophobicity (Wagner *et al.*, 2000). Similar results were observed for NB, except that P2, showing higher solubilities, also showed lower hydrophobicity than the finer concentrates. YP P1 resulted in higher solubility, which also showed lower surface hydrophobicity and lower zeta potential than P2. CH P1 and P2 solubilities showed no significant differences. Regarding proteins extracted at different f:w ratios, no differences were found.

3.3.4.2. WHC and OHC

WHC results, defined as the amount of water that 1 g of protein can absorb, are shown in Table 3.4. WHC and the affinity of water to bind to the proteins through electrostatic interactions are related to protein structure and the hydrophilic groups readily present to interact with water (Lam *et al.*, 2018). No significant effects were observed for the different f:w ratios on WHC for each pulse. NB showed higher WHC than the other pulses. NB also showed the highest solubility values, which was in agreement with lower L/V ratios for this pulse. WHC of protein concentrates obtained from P2 flours also showed NB to have the highest WHC, followed by GL, YP, and CH. Comparison between particle sizes showed no difference in WHC between concentrates, except for CH where concentrates obtained from P1 showed higher WHC than those obtained from P2, possibly due to a lower L/V ratio and hydrophobicity results.

Table 3.4. Protein solubility (%), water hydration capacity (WHC) and oil holding capacity (OHC) of protein concentrates extracted at different f:w ratios for different pulse types and flour particle sizes (P1 and P2). Data represents the mean ± one standard deviation (n=6).

	% Protein Solubility		
Pulse/Size	**1:10**	**1:7**	**1:5**
YP P1	70.01 ± 2.05 ^{Aa}	65.25 ± 4.80 ^{a}	68.82 ± 2.14 ^{a}
YP P2	61.62 ± 3.12 ^{B}	N/A	N/A
GL P1	59.04 ± 3.67 ^{Ba}	53.21 ± 5.18 ^{a}	60.32 ± 6.86 ^{a}
GL P2	72.16 ± 5.27 ^{A}	N/A	N/A
CH P1	70.70 ± 3.46 ^{Aa}	69.79 ± 2.90 ^{a}	70.23 ± 2.86 ^{a}
CH P2	74.54 ± 5.96 ^{A}	N/A	N/A
NB P1	78.29 ± 2.70 ^{Ba}	72.07 ± 4.41 ^{ab}	69.53 ± 4.96 ^{b}
NB P2	87.01 ± 3.79 ^{A}	N/A	N/A
	WHC (g/g)		
Pulse/Size	**1:10**	**1:7**	**1:5**
YP P1	2.32 ± 0.28 ^{Aa}	2.18 ± 0.05 ^{a}	2.49 ± 0.19 ^{a}
YP P2	2.15 ± 0.09 ^{A}	N/A	N/A
GL P1	2.30 ± 0.09 ^{Aa}	2.25 ± 0.02 ^{a}	2.16 ± 0.08 ^{a}
GL P2	2.28 ± 0.07 ^{A}	N/A	N/A
CH P1	2.43 ± 0.22 ^{Aa}	2.64 ± 0.03 ^{a}	2.40 ± 0.14 ^{a}
CH P2	1.98 ± 0.15 ^{B}	N/A	N/A
NB P1	2.83 ± 0.22 ^{Aa}	2.83 ± 0.18 ^{a}	2.98 ± 0.05 ^{a}
NB P2	2.68 ± 0.06 ^{Aa}	N/A	N/A
	OHC (g/g)		
Pulse/Size	**1:10**	**1:7**	**1:5**
YP P1	1.87 ± 0.07 ^{Aa}	1.78 ± 0.06 ^{a}	1.77 ± 0.12 ^{a}
YP P2	1.81 ± 0.11 ^{A}	N/A	N/A
GL P1	1.86 ± 0.12 ^{Aa}	1.91 ± 0.07 ^{a}	1.88 ± 0.04 ^{a}
GL P2	1.73 ± 0.05 ^{A}	N/A	N/A
CH P1	1.93 ± 0.02 ^{Aa}	1.96 ± 0.13 ^{a}	1.93 ± 0.02 ^{a}
CH P2	1.54 ± 0.06 ^{B}	N/A	N/A
NB P1	2.18 ± 0.11 ^{Aa}	2.04 ± 0.12 ^{a}	2.13 ± 0.19 ^{a}
NB P2	1.80 ± 0.07 ^{B}	N/A	N/A

Abbreviations: YP, yellow pea; GL, green lentil; CH, chickpea; NB, navy bean. P1- Screen size 0.5 mm, P2-Mesh size 1.27 mm. *Note*: NA (not applicable). Lowercase letters show significant differences within pulse type for different f:w ratios ($p < 0.05$). Uppercase letter shows significant differences between different particle sizes of the same pulse ($p < 0.05$).

WHC values were found to be in the range of what was obtained in previous studies from isolates obtained from AEIP (Stone *et al.*, 2015a)

OHC results, defined as the amount of oil that 1 g of protein can entrap, are shown in Table 3.4. OHC can be affected by pulse variety and the extraction method used for protein isolation as these factors affect the hydrophobic interactions between the oil and nonpolar groups present in the protein (Lam *et al.*, 2018). Results were similar for the concentrates obtained with different f:w ratios within pulse type, for all pulses. The OHC of concentrates extracted from P2 flours ranged from 1.54 to 1.81 g/g while those obtained from P1 ranged from 1.77 to 2.18 g/g. OHC values for pulses were reported to be between 1.0 and 3.96 g/g for different pulses (Siddiq & Uebersax, 2012; Singhal *et al.*, 2016).

3.3.4.3. Emulsifying properties

Results for EAI, reported as the protein ability of stabilizing the interfacial area in m^2/g of protein, and ESI, reported as the stability of the formed emulsion over time, are shown in Table 3.5. The results showed that a decrease in the f:w ratios used for extraction resulted in similar EAI for YP and CH protein concentrates. For GL and NB, EAI resulted in higher values for the concentrates extracted using the 1:10 ratio and these were the highest values among all pulses. Shevkani *et al.*, (2015) found higher EAI values when L/V ratios decreased, reasoning that vicilin presented higher flexibility and lower molecular weight allowing better migration and accommodation of the proteins at the interface, which could explain the high EAI values. Can Karaca *et al.*, (2011b) reported higher EAI values at pH 7.0 (~42–48 m^2/g) for pea, lentil, and chickpea protein isolates prepared by AEIP than those obtained in the present research which ranged from ~26-32 m^2/g. Chang *et al.* (2015) found that proteins with higher solubilities allow the proteins to migrate to the oil- water interface and rearrange at the interface. However, YP and GL protein concentrates extracted from P2 flours had significantly lower EAI than those extracted from P1 flours, despite their solubilities being higher for YP P1 but lower for GL P1. However, the surface hydrophobicities for P2 for these samples were higher than P1 isolates, where a higher hydrophobicity will contribute more hydrophobic residues on the surface of the proteins to orient towards the oil phase (Papalamprou *et al.*, 2010).

Table 3.5. Emulsifying activity index (EAI), emulsifying stability index (ESI) (%), foam capacity (FC) and stability (FS) values of protein concentrates extracted at different f:w ratios using AEIP for two different particle size pulse flours (P1 and P2). Data represent the mean ± one standard deviation (n=6).

Pulse/Size	1:10	1:7	1:5	1:10	1:7	1:5
		EAI (%)			**ESI (%)**	
YP P1	26.65 ± 1.30 ^{Aa}	26.53 ± 1.21 ^{a}	25.16 ± 1.18 ^{a}	13.26 ± 0.30 ^{Aa}	12.39 ± 0.36 ^{b}	12.79 ± 0.44 ^{ab}
YP P2	19.96 ± 1.87 ^{B}	N/A	N/A	12.22 ± 0.82 ^{B}	N/A	N/A
GL P1	30.95 ± 2.01 ^{Aa}	29.69 ± 0.74 ^{ab}	27.67 ± 1.04 ^{b}	11.75 ± 0.34 ^{Bb}	12.42 ± 0.55 ^{ab}	13.55 ± 1.65 ^{a}
GL P2	25.53 ± 1.65 ^{B}	N/A	N/A	12.26 ± 0.22 ^{A}	N/A	N/A
CH P1	27.10 ± 1.67 ^{Aa}	28.19 ± 1.06 ^{a}	25.58 ± 4.28 ^{a}	12.49 ± 0.58 ^{Aa}	13.17 ± 0.40 ^{a}	12.95 ± 1.00 ^{a}
CH P2	24.94 ± 3.17 ^{A}	N/A	N/A	13.06 ± 1.05 ^{A}	N/A	N/A
NB P1	31.69 ± 1.65 ^{Aa}	26.01 ± 1.44 ^{b}	27.29 ± 1.17 ^{b}	12.11 ± 0.65 ^{Ba}	13.01 ± 1.07 ^{a}	12.48 ± 1.17 ^{a}
NB P2	32.42 ± 0.84 ^{A}	N/A	N/A	14.04 ± 0.94 ^{A}	N/A	N/A
		FC(%)			**FS (%)**	
YP P1	297.14 ± 26.63 ^{Aa}	263.33 ± 18.74 ^{b}	256.19 ± 14.33 ^{b}	87.75 ± 6.31 ^{Aa}	86.49 ± 4.11 ^{a}	87.68 ± 5.42 ^{a}
YP P2	264.44 ± 23.35 ^{B}	N/A	N/A	90.73 ± 1.32 ^{A}	N/A	N/A
GL P1	291.11 ± 18.70 ^{Ab}	322.22 ± 10.04 ^{a}	241.11 ± 21.67 ^{c}	85.51 ± 11.22 ^{Aab}	75.43 ± 4.70 ^{b}	88.46 ± 4.02 ^{a}
GL P2	280.00 ± 5.96 ^{A}	N/A	N/A	88.48 ± 1.11^{A}	N/A	N/A
CH P1	306.67 ± 19.44 ^{Aa}	306.67 ± 13.33 ^{a}	295.00 ± 24.38 ^{a}	83.00 ± 7.90 ^{Aab}	77.16 ± 1.67 ^{b}	86.99 ± 5.75 ^{a}
CH P2	294.44 ± 23.63 ^{A}	N/A	N/A	89.56 ± 1.82 ^{A}	N/A	N/A
NB P1	300.95 ± 13.57 ^{Aa}	291.25 ± 4.93 ^{a}	283.33 ± 13.82 ^{a}	85.05 ± 8.80 ^{Aa}	89.41 ± 4.93 ^{a}	87.44 ± 3.83 ^{a}
NB P2	252.22 ± 18.58 ^{B}	N/A	N/A	88.15 ± 1.79 ^{A}	N/A	N/A

Abbreviations: YP, yellow pea; GL, green lentil; CH, chickpea; NB, navy bean. P1- Screen size 0.5 mm, P2-Mesh size 1.27 mm.. *Note*: NA (not applicable) . Lowercase letters show significant differences within pulse type for different f:w ratios ($p < 0.05$). Uppercase letter shows significant differences between different particle sizes of the same pulse ($p < 0.05$)

ESI results for the protein concentrates extracted at all f:w ratios for P1 were similar in magnitude ranging from 11.75 to 13.55 min, with YP and CH showing greater stability compared to GL and NB. Can Karaca *et al.* (2011b) reported higher ESI values for proteins with higher surface charges, which could lead to increased emulsion stability due to electrostatic repulsion between droplets. Lower zeta potential values were found for both YP and CH, which could explain higher stabilities of the emulsions. The ESI values at pH 7 obtained for black, red kidney, great northern, and pinto bean protein isolates prepared by salt extraction were ~39−52min at pH 7 (Hojilla-Evangelista *et al.*, 2018) and higher than those obtained in the present study. However, Stone *et al.* (2015b) reported that both extraction method and cultivar were significant factors affecting emulsion stability. Stone *et al.* (2015a) reported similar ESI values (~10.9−11.2 min) for pea protein isolates extracted and evaluated under the same conditions as the present results.

3.3.4.4. Foaming properties

Foaming capacity results, referring to the amount of foam formed when protein is added to the system, are presented in Table 3.5. The ability of protein to be a good foaming agent is affected by its flexibility to rearrange at its interface, its hydrophobicity through the ability to orientate hydrophobic residues towards the air phase, and its migration to the interface which relates to the protein solubility (Damodaran *et al.*, 2007) FC values at pH 7.0 ranged from ~256% to 297% for YP, ~241% to 322% for GL, ~294% to 307% for CH, and ~283% to 300% for NB. FC values from previous research were different, but the methodology (protein concentration, equipment, etc.) used was not consistent across studies, which can affect the results (Aluko *et al.*, 2009). Stone *et al.* (2015b) found lower values for pea protein isolates extracted using the same method and protein concentration (~155%−184%), while Lam (2016) reported values similar to the ones found in the present study ranging from ~177% to 243% for different pea cultivars grown under different environments over two different growing seasons when tested under the same conditions. Lee *et al.* (2021) found lower FC values for lentil proteins from different countries extracted under similar conditions, but the protein solutions were in lower concentrations than in the present study. Martinez *et al.* (2016) found values that ranged from 122% to 154% for faba bean protein concentrates tested using 1% w/w protein solutions as used in the present study.

No significant effect on different extraction f:w ratios were found for NB or CH, while YP and GL had significantly higher FC for the concentrates extracted from 1:10 and 1:7. Shevkani *et al.*

(2019) reported that higher protein solubility resulted in greater FC, however, no differences in solubility were found for YP or GL concentrates extracted using different f:w ratios. The effect of different particle-sized flours on protein extraction showed higher FC for concentrates from YP and NB prepared from P1 flours. A possible explanation for YP could be that P1 showed higher solubility than P2. In the case of NB, though the finer flour didn't show higher solubility than the coarser particle-sized flour, it presented higher hydrophobicity which could explain higher affinity towards the interface, resulting in higher foam formation. de la Rosa-Millán *et al.* (2018) reported that the ratio of alpha helixes and beta sheets decreased with alkaline protein extraction, resulting in higher foaming and emulsion properties favoured by higher flexibility of the molecule.

FS, which refers to how well the foam is held due to the addition of the protein over 30 min, was also measured (Table 3.5). The presence of the protein will act as a surfactant, reducing the interfacial tension of the two involved phases and aiding in forming a thick film surrounding the gas bubbles to prevent rupture (Konieczny *et al.*, 2020). FS values for P1 concentrates ranged from ~86% to 88% for YP, ~75% to 88% for GL, ~77% to 87% for CH, and ~85% to 89% for NB. The loss in volume after 30 min for all 1:10 P1 samples was ~2.5%–2.9%. After 30 min of foam formation, Lee *et al.* (2021) found similar FS values for lentil protein foams (~80%–85%), while FS values determined by Martinez *et al.* (2016) and Shevkani *et al.* (2015) ranged from ~71%–80% for faba beans and 87%–132% for field pea, respectively. No differences in FS were found for foams prepared with concentrates extracted from P1 and P2 particle-size flours.

3.4. Conclusions

This study explored the effect of different f:w ratios and two different particle-sized flours on the protein extraction yields and functional properties of the resulting protein concentrates. The results have shown that a reduction in the water used for protein extraction led to a significant decrease in the protein yields. However, extractions performed with a smaller particle size (P1) of pulse flours, using the same solute-to-solvent ratio, can lead to an increase in the extracted protein yield. This suggests that control of the particle size of the pulse flour produced could help in the extraction step of protein concentrate/ isolate production through AEIP. A future study could also address extractions at 1:7 and 1:5 f:w ratios with P1 and P2 particle sizes to determine if a reduction in particle size achieves the same yields with reduced water, compared to the most commonly 1:10 f:w ratio used in protein extraction. Functional properties showed no major differences between

proteins extracted at different f:w ratios, while differences were found between pulse types. In some cases, concentrates extracted from P2 and P1 flours differed for certain functional properties, but this was not consistent for all pulses.

3.5. Connection to the next study

This study showed that reducing water use during protein extraction can compromise the extraction and protein yields. However, reducing the particle size of the starting flours can be an advantageous approach to increase the protein contents of the isolates. Knowing the parameters that worked best for the extraction of proteins (f:w ratio of 1:10 and 0.5 mm mesh size sieved flour), the next study will evaluate these parameters on two different extraction methods that are commonly used for the extraction of proteins: salt extraction-dialysis and alkaline extraction – isoelectric precipitation. Assessing yields and examining how the extraction method affect the functionality of the resulting proteins is crucial for understanding how proteins can be customized for specific purposes.

4. FUNCTIONALITY AND NUTRITIONAL PROPERTIES OF YELLOW PEA, GREEN LENTIL, CHICKPEA, AND NAVY BEAN PROTEINS EXTRACTED BY DIFFERENT METHODS[2]

4.1. Abstract

The effect of two different extraction methods (alkaline extraction isoelectric precipitation—AEIP and salt extraction dialysis—SE) on the production of protein isolates from four different pulse types grown in Canada (yellow pea-YP, green lentil-GL, chickpea-CH and navy bean-NB) were studied for their physicochemical, functional, and nutritional properties. The extraction method and type of pulse had an effect on extraction and protein yields. The AEIP method resulted in higher protein yields for CH and GL. In contrast, YP and NB protein yields achieved were higher with the SE method. The method used for extraction had an influence on the surface properties and the functionality of the isolated proteins. SE-produced isolates presented higher solubility, foaming capacity and emulsion activity than those prepared through AEIP. Protein quality results showed that higher *in-vitro* protein digestibility (IVPD) and *in-vitro* protein digestibility corrected amino-acid scores (IV-PDCAAS) were achieved for proteins obtained through AEIP than those prepared for SE samples. The current study contributes to a better understanding of the effects of extraction methods on the resulting protein characteristics and properties of different pulses grown in Canada, which can impact their suitability for different product applications.

4.2. Introduction

Pulses like peas, lentils, chickpeas or common beans are leguminous crops commonly grown in the Canadian Prairies and they serve as plant-based sources of protein (Stone *et al.*, 2019). The increase in the demand for novel plant protein ingredients, and their economic importance, has led

[2] Higa, F., House, J. D., & Nickerson, M. T. (2023). Functionality and nutritional properties of yellow pea, green lentil, chickpea, and navy bean proteins extracted by different methods. European Food Research and Technology, 1, 3. https://doi.org/10.1007/s00217-023-04385-9

to the search for extraction methods with high yields and desired functional properties highly sought after in the food industry. Pulse proteins can be extracted by dry or wet methods. Air classification can achieve enriched protein flours with a protein content between ~ 40–65%, while proteins extracted through wet extraction methods can yield higher protein contents reaching ~ 70–90% protein (Singhal *et al.*, 2016). Among these methods, the most common ones are the solubilization of proteins at alkaline pH followed by precipitation of the proteins at the isoelectric point (AEIP) and solubilization of proteins in a salt solution (SE) followed by a second step to remove the salts (*e.g.*, dialysis, ultra- filtration, dilution) (Stone *et al.*, 2015b). The main storage proteins in pulses, comprising around 70–80%, are globulins (salt soluble) followed by albumins 10–20% (water-soluble). Other proteins, such as prolamins and glutelins, are also found in pulses but in lower amounts (Boye *et al.*, 2010; Chang *et al.*, 2022). The major proteins present, globulins and albumins, are responsible for the resulting functional properties that these proteins can exhibit, like foaming or emulsifying properties. The main globulins found are 11*S*-hexameric legumins and 7*S*-trimeric, glycosylated vicilins (Chang *et al.*, 2012; Tanger *et al.*, 2020). Legumins are comprised of ~ 40 and ~ 22 kDa subunits, while vicilin presents subunits of ~ 70, ~ 50, ~ 33–35, and ~ 20 kDa. Albumins comprise ~ 10–12 kDa (Chang *et al.*, 2012) and ~ 6–26 kDa (Hall *et al.*, 2017) polypeptides. Considering the different solubility of pulse proteins in different solvents (Singhal *et al.*, 2016), selecting an extraction method for proteins will also allow targeting the predominant proteins to be extracted while accounting for changes that the proteins might undergo due to the extraction process. For example, the salt extraction method (SE) extracts a mixture of globulins and albumins, while the alkaline extraction–isoelectric precipitation method (AEIP) mainly extracts globulins (Stone *et al.*, 2015a). The difference in extraction conditions can also affect the end functionality that they will exhibit. For example, Osemwota *et al.* (2022) studied the functionality of albumin, globulin and glutelin fractions of green lentils. The authors found that the globulin fraction presented lower oil holding capacity (OHC) than the albumin fraction, while albumins reported higher foaming capacity than globulin or glutelin fractions at pH 7 and 9. Ghumman *et al.* (2016) reported similar results for foaming capacity (FC) of the albumin fraction of lentil and horse gram proteins. Similarly, Can Karaca *et al.* (2011b) observed that the solubility of proteins was influenced by the extraction method used, with higher solubilities found in proteins isolated using the AEIP method compared to those obtained through the SE method. These

differences were attributed to the resulting changes in the surface properties of the extracted proteins induced by the extraction methods used.

The consumption of pulses has been linked to various beneficial health effects, such as reducing the risk of developing type II diabetes and certain types of cancer. In addition, incorporating pulses into the diet can help decrease the risk of cardiovascular diseases and cholesterol (Carbas *et al.*, 2021). However, pulses are deficient in sulphur-containing amino acids (cysteine and methionine) and tryptophan. Their protein digestibility, referring to their ease to be hydrolyzed by proteases, is lower compared to proteins from animal sources. Protein digestibility can be affected by the protein structure, denaturation (*e.g.*, thermal or processing history of the product), and anti-nutritional compounds (*e.g.*, enzyme inhibitors, phytates, phenolics, etc.) (Sá *et al.*, 2020). Different protein extraction methods subject proteins to different treatment conditions which can influence their structure, composition and type of proteins being extracted. These factors ultimately impact the susceptibility of proteins to enzymatic hydrolysis.

The aim of this study was to extract proteins from four different pulse types, yellow pea (YP), green lentil (GL), kabuli chickpea (CH) and navy bean (NB), using two different extraction methods (AEIP and SE). The extracted proteins were evaluated for their physicochemical, functional, and nutritional properties to assess the impact of the extraction method on these parameters. Additionally, the determination of total phenolics (TPC) was conducted to potentially explain variations in the extraction yields achieved by different extraction methods. The findings of this study will contribute to a better understanding of how the choice of protein extraction method affects the resulting functionality and nutritional aspects of the proteins.

4.3. Materials and Methods

4.3.1. Materials

Four different pulses harvested in the 2018 crop year were used in this study. Navy bean (Nautica) was purchased from Hensall Co-op (Hensall, ON), while yellow pea (CDC Spectrum), green lentil (CDC Greenstar) and Kabuli chickpea (CDC Orion) were obtained from Reisner Farm Ltd. (Limerick, SK). Flours were prepared with a hammer mill for seed pre-breaking (Jacobson Model 120- B, Jacobson Machine Works Inc., IA, USA), followed by milling with a knife mill using a 0.5 mm screen (Ferkar 5 Model, KFM, d.o.o., Slovenia). Particle size distributions of the flours were $D_{[3,4]}$ of 85.7 μm for YP, 120.0 μm for GL, 62.8 for CH, and 141.9 μm for NB (Malvern

Master- sizer 2000 with Scirocco 2000 accessory, Malvern Instruments Inc, Westborough, MA). Protein and moisture were determined by the official methods AOAC Official Method 920.87 and 925.10 (AOAC, 2003).

4.3.2. Protein extraction

Samples were defatted before extraction and extracted following the procedure used by Stone *et al.* (2015b) using hexane (1:3 w/v) for 40 min at 500 rpm. Samples were filtered using Whatman #1 paper (Whatman International Ltd., Maidstone, United Kingdom), and three subsequent defatting cycles were performed. The flour was recovered and dried in the fume hood overnight before being used for extraction. The water used in all extractions and solutions for further determination was Milli-Q^{TM} water (Millipore Corporation, MA, USA). The protein and moisture of the protein isolates were determined by the official methods AOAC Official Method 920.87 and 925.10 (AOAC, 2003).

4.3.3. Alkaline extraction

Alkaline extraction followed by isoelectric precipitation (AEIP) was performed in triplicates following the procedure by Stone *et al.* (2015b). Flours were dispersed in a 1:10 flour: water (f:w) ratio; the pH was adjusted at 9.5 using 2M NaOH and stirred for 1 h at 500 rpm. After solubilization of proteins at alkaline pH, the samples were centrifuged at 4500 × g for 20 min at 4 ºC (J-E Avanti Centrifuge Beckman Coulter, CA, USA) to separate the solubilized proteins from other insoluble components. The supernatant was collected, and the pH was adjusted to 4.5 using 2N HCl and stirred at 500 rpm for 30 min. The precipitated proteins were centrifuged at 4500 × g for 20 min at 4 ºC and stored at – 30 ºC in aluminium pans (VWR, Mississauga, ON, Canada) until they were freeze-dried (Labconco Freezone 18L freeze-dryer system, Labconco, Kansas City, MO, USA).

4.3.4. Salt extraction

The salt extraction-dialysis (SE) was performed in triplicates following the procedure described by Stone *et al.* (2015b). Flour was dispersed in a 0.1 M sodium phosphate buffer with 6.4% KCl at a 1:10 (f:w) ratio and stirred at room temperature for 24 h at 500 rpm. The samples were centrifuged at 4500 × g for 20 min at 4 ºC . The pellet containing insoluble fractions was discarded and the supernatant was dialyzed (6–8 kDa Spectra dialysis membranes, Spectrum

laboratories, Rancho Dominguez, CA, USA) against distilled water for ~ 72 h at 4ºC until the conductivity reached ~ 20 micro siemens. The distilled water was changed every 8 h daily. The extracted proteins were stored at – 30ºC and later freeze-dried (Labconco Freezone 6 freeze dryer, Labconco, Kansas City, MO, USA).

4.3.5. Physicochemical properties

4.3.5.1. Zeta potential

The surface charge of the protein isolates was determined using a Zetasizer Nano-ZS90 analyzer (Malvern Instruments, Westborough, MA, U.S.A.) following the procedure by Can Karaca *et al.* (2011b). Electrophoretic mobility was determined on 0.05% (w/w) protein solutions stirred for 1h at pH 7.0 adjusted using 0.1 M NaOH or HCl. Electrophoretic mobility was converted to zeta potential (ζ) using Henry's equation:

$$U_E = \frac{2\varepsilon\,\zeta\,f(\kappa\alpha)}{3\eta} \tag{4.1}$$

where ε is the permittivity of the medium (Farad/m), (κα) is a function of the particle radius (α, nm) and Debye length (κ) and η is the viscosity (mPa·s).

4.3.5.2. Surface Hydrophobicity

Surface hydrophobicity was determined using the 8-anilino-1-naphtalensulfonic acid (ANS) - fluorescent probe method following the procedure by Kato & Nakai (1980) using a Fluoromax-4 (Horiba Jobin Yvon Inc., Edison, NJ, USA). In brief, 0.25% (w/w) protein solutions were stirred for 1 h at pH 7. Dilutions of the protein solution were prepared at 0.05, 0.10, 0.15 and 0.20%. To 1.6 mL of each protein solution, 20 µl of ANS was added and vortexed for 10 s. The samples were incubated for 5 min in the dark and measured at 390 nm excitation and 490 nm emission with a slit width of 1 nm. Protein solution blanks were also prepared, where 20 µL of water were added instead of ANS, vortexed and fluorescence intensity (FI) was measured under the same conditions. Protein hydrophobicity measurements were calculated from the slope of FI of protein with ANS minus protein blanks against concentration.

4.3.5.3. Legumin/vicilin-albumin/globulin ratios

Sodium dodecyl sulphate polyacrylamide (SDS-PAGE) gels were performed on the extracted proteins under reducing conditions following the method by Laemmli (1970) with some modifications. Legumin/vicilin ratios (L/V ratio) and the albumin/globulin ratios (A/G) were determined. A 15% separating gel (pH 8.6) and a 4% stacking gel (pH 6.8) were used. In brief, 35 µL of a 1% protein solution were added to 35 µL of sample buffer containing 20 mM Tris–HCl buffer pH 7.6, 10% SDS solution, 2% β-mercaptoethanol, 50% (v/v) glycerol and 0.01% bromophenol blue. The samples were vortexed for 10 s and heated in a water bath (Precision Microprocessor water bath, Thermo Fisher, Massachusetts, USA) at 80 ºC for 10 min. After incubation, the samples were centrifuged at 12,100 × g for 10 min and loaded into the gel. An MGV-202 Vertical Mini-Gel System (CBS Scientific, San Diego, CA, USA) was used to run the samples for ~ 1.5 h at 120 V using a Power Source 300 V Electrophoresis Power Supply (VWR, Mississauga, ON, Canada). A pre-stained BLUelf protein ladder was used as a molecular weight marker (FroggaBio Scientific Solutions, Toronto, Ontario, Canada) that ranged from 5 to 245 kDa. The gels were fixed overnight with a fixing solution (5:1:4 methanol: glacial acetic acid: water), followed by a 4 h staining using a 0.1% Coomassie blue solution. Afterwards, the gels were distained for 4 h using a 3:6:1 methanol: water: glacial acetic acid (v:v:v) solution. The protein bands were identified using the molecular weight standard and quantified by densitometry using the ImageJ software (National Institutes of Health Bethesda, Maryland, USA).

4.3.5.4. Total phenolic content (TPC)

Total phenolic content was determined using the Folin Ciocalteau method following the procedures by Konieczny *et al.* (2020) and Cacique *et al.* (2021), with some modifications. Firstly, phenolic extraction was carried out with an extraction solvent consisting of 1% (v/v) of methanol in HCl. Five grams of sample were added to 50 mL capped centrifuge tubes, and 5 mL of the extraction solvent were added and placed in a shaker for 2 h. Afterwards, samples were centrifuged at 1050 ×g for 10 min. The supernatant was collected, and the extraction was repeated twice, adding 5 mL of extraction solvent each time. The supernatant of the three successive extractions was pooled and kept at -18 ºC until phenolics were measured. Extractions were determined in duplicates. Phenolic determination was done by adding 0.1 mL of sample to 1.3 mL of milli-Q water, followed by 0.1 mL of the Folin reagent and 1 mL of sodium carbonate. The samples were

vortexed for 10 s and left in the dark for 1.5 h. Measurements were performed at 725 nm (Genesys 10 UV Scanning Spectrophotometer, Thermo Fisher Scientific Inc.). A gallic acid standard curve with 100–500 µg/mL concentrations was prepared, and concentrations of TPC in the sample were calculated from the curve. Results were expressed as mg gallic acid/g protein.

4.3.6. Functional properties

4.3.6.1. Solubility

Protein solubility was determined following the procedure by Morr *et al.* (1985), where 0.2 g of protein was dispersed in ~ 19 mL of milli-Q water and the pH was adjusted to 7.0 using 0.1 NaOH or HCl and stirred at 500 rpm for 1 h. Afterwards, the solutions were adjusted to 20 g total and centrifuged at 4180 ×g for 10 min (Sorvall ST8 Centrifuge, Thermo Scientific, Massachusetts, USA). The supernatant was collected, and the protein content was determined using a micro-Kjeldahl digestion and distillation unit (RapidStill 1, Labconco, Kansas City, MO, USA). A factor of 6.25 was used to convert nitrogen to protein. The % solubility was calculated as the amount of protein present in the supernatant, divided by the total protein present in the sample.

4.3.6.2. Emulsion activity and stability index (EAI – ESI)

Emulsion activity and stability were evaluated following the method by Pearce & Kinsella (1978) with some modifications. Protein solutions (0.25% w/w) were prepared, and the pH was adjusted to 7.0 using 0.1M NaOH or HCl and stirred for 1 h at 500 rpm. Emulsions were prepared using 5 mL of protein solution and 4 g of canola oil in 50 mL centrifuge tubes, using an IKA homogenizer (IKA Works, T-10 basic ULTRA-TURRAX® Wilmington, NC, USA) with a S10 N dispersion tool, at speed 5 (12,800 rpm) for 5 min. Immediately after homogenization, a 50 µL aliquot from the bottom of the tube was sampled and added to 7 mL of sodium phosphate buffer with 0.1% SDS (pH 7.0) and vortexed for 10 s. The absorbance was measured at 500 nm. After 10 min, another 50 µL aliquot was sampled, and the measurement was repeated to calculate ESI.
EAI was calculated as follows: c is the solution's concentration (in g/mL), F is the dilution factor, φ is the oil/total volume ratio, and A_0 is the initial absorbance.

$$EAI\ (\frac{m^2}{g}) = \frac{2 \times 2.203 \times A_0 \times N}{c \times \varphi \times 10000} \quad (4.2)$$

ESI was calculated using Eq. (4.3), where ΔA is the difference between A_0, the initial absorbance, and A_f the absorbance of the emulsion measured after t = 10 min.

$$ESI\ (\text{min}) = \frac{A_0}{\Delta A} \times t \tag{4.3}$$

4.3.6.3. Foaming capacity and stability (FC – FS)

Foaming capacity and stability were prepared following the method by Stone *et al.* (2019) with some modifications. Protein solutions (1% w/w) were prepared, and the pH was adjusted to 7.0 using 0.1 M NaOH and HCl and stirred for 1 h at 500 rpm. Foams were prepared by homogenizing 15 mL of protein solution in 400 mL beakers for 5 min at speed 5 (12,800 rpm) using an IKA homogenizer (T-10 Basic ULTRA-TURRAX; IKA Works, Wilmington, NC, USA) with an S10 N dispersion tool. The foam was transferred immediately to 100mL graduated cylinders, and the formed foam was measured. Foaming capacity was calculated as follows Eq. (4.4):

$$\%FC = \frac{V_0}{V_l} \times 100 \tag{4.4}$$

where V_f is the volume of foam, and V_l represents the initial volume of protein solution (15 mL). After 30 min, the foam height was recorded, and foaming stability was calculated as shown in Eq. (4.5)

$$\%FS = \frac{V_f}{V_0} \times 100 \tag{4.5}$$

where $V_{f\,30}$ is the volume of foam after 30 min, and V_f is the initial foam volume.

4.3.6.4. Water hydration and oil holding capacity (WHC – OHC)

Water hydration and oil holding capacities were performed following the method by Stone *et al.* (2019) with some modifications. Firstly, 0.25 g of protein was weighed in 50 mL centrifuge tubes. The weight of the tube and the sample were recorded, and 5 g of water or oil was added to the tube. Each tube was vortexed for 10 s every 5 min for 30 min, followed by centrifugation at

1000 ×g for 15 min. The supernatant was discarded, and the pellet weight was recorded. WHC and OHC were calculated as a percentage of the weight gained divided by the weight of the dry sample.

4.3.7. Protein quality

Amino acid analysis was determined by the AOAC Official Method 982.30 for all amino acids (except methionine, cysteine, and tryptophan) through acid hydrolysis with 6 N for 24 h. Methionine and cysteine were determined following the AOAC Official method 985.28 performed through acid oxidation and acid hydrolysis (AOAC, 2005). Tryptophan content was determined following the ISO protocol 13,904 (ISO, 2016). The hydrolysates amino acid profiles were determined using an AccQ-Tag Ultra C18, 1.7 µm column on a Shimadzu UPLC system (Kyoto, Japan), determined using the AccQ-Tag Ultra protocol (Astephen, 2018). Determinations were run once, and a casein standard was run along with the samples.

The amino acid score, designating the limiting essential amino acid, was calculated as the ratio of mg aa/g protein divided by a reference protein (FAO 1991) established as the amino acid reference pattern for children from 2–5 years old (tryptophan 11, lysine 58, histidine 19, phenylalanine and tyrosine 63, leucine 66, isoleucine 28, methionine and cysteine 25, valine 35 and threonine 34) (FAO 2013).

In-vitro protein digestibility was determined following the multienzyme method by Hsu *et al.* (1977). Preparation of the enzyme stock solution consisted in 31 mg of chymotrypsin (bovine pancreas P40 units/mg protein), 16 mg of trypsin (porcine pancreas 13,000–20,000 BAEE units/mg protein), 13 mg of protease (*Streptomyces griseus*, P3.5 units/mg) and 10 mL of water. The enzyme solution was placed in a water bath at 37 ºC while stirring, and the pH was adjusted to 8.0 with NaOH/ HCl 0.1 M. For the sample preparation, 62.5 mg of protein sample were weighed in glass vials, 10 mL of Milli-Q water added and placed in a water bath at 37ºC for 1 h while stirring. The pH of the protein solution was also adjusted to 8.0 with 0.1 M NaOH or HCl followed by adding 1 mL of the enzyme stock solution (at pH 8.0). The initial pH before enzyme addition and the pH every minute for 10 min were recorded. IVPD was calculated as follows:

$$IVPD = 65.66 + 18.10 \times \Delta pH \quad (4.6)$$

The drop in pH (ΔpH) represents the difference between the initial and the final pH measurements.

In-vitro protein digestibility corrected amino acid score (IV-PDCAAS) was calculated by multiplying the limiting amino acid score and the *in-vitro* protein digestibility.

4.3.8. Statistical analysis

Protein extractions were performed in triplicates for each pulse ingredient. Data analysis was performed using R software (R Core Team 2021, Vienna, Austria). A one-way analysis of variance (ANOVA) with Tukey's posthoc test was performed to find differences between the samples. A two-way ANOVA was conducted to investigate the interactions between the extraction method and the pulse type for all the properties evaluated. Pearson's test was also performed to find existing correlations. All determinations were performed in duplicates on each triplicate extraction, and results are informed as the mean ± the standard deviation (SD).

4.4. Results and discussion

4.4.1. Extraction and protein yields

The starting flours and isolates' protein contents along with their yields are shown in Table 4.1. The protein contents reported for the extracted isolates using AEIP ranged from 89.3% for YP to 81.6% for NB. The SE sample protein contents were highest for CH at 87.7% and the lowest at 83.3% for NB. A two-way analysis of variance showed that the extraction method did not significantly affect the final protein content ($p > 0.05$). Results from two-way ANOVA indicating the significance of the main factors and their interaction on all the properties evaluated can be found in Appendix A. Previous studies found that the AEIP method achieved higher protein contents when compared to other extraction methods, such as SE-dialysis or SE followed by ultrafiltration, attributing these results to higher solubilities of carbohydrates in dilute salt solutions (Yang *et al.*, 2021). Opposite results were found in a study by Can Karaca *et al.* (2011a) where SE-extracted isolates from canola had higher protein contents than isolates prepared by AEIP, where possibly the isoelectric point of the different proteins in canola may have influenced the precipitation of the proteins.

The type of pulse ($p < 0.001$) and the interaction between extraction method and pulse ($p < 0.001$) were found to be significant factors influencing the protein content of the extracted isolates. The extracted protein isolates exhibited higher protein contents for YP, and lowest for NB. Can Karaca *et al.* (2011b) reported protein contents for chickpea (~ 82–85%), lentil (~ 82–75%) and

pea (~ 89–81%) in the range of those found in this study. Protein contents of navy bean concentrates prepared by AEIP were reported to have between 80–83% protein for alkaline extracted proteins (Kohnhorst *et al.*, 1991).

Table 4.1. Protein contents (dry basis, d.b.) of the flours and protein isolates, extraction and protein yields (%) and total phenolic content (TPC; mg gallic acid equivalents (GAE)/ g protein) of protein isolates prepared using alkaline extraction – isoelectric precipitation and salt extraction-dialysis.

Pulse	Protein content in the flours (%, d.b.)	Protein content in protein isolates (%, d.b.)	Extraction yield (%, d.b.)	Protein yield (%, d.b.)	TPC (mg GAE/ g protein)
a)	*Proteins extracted using alkaline extraction – isoelectric precipitation (AEIP)*				
YP	21.31 ± 1.03 ^{a}	89.35 ± 0.51 ^{a}	17.7 ± 0.1 ^{cd}	72.2 ± 0.3 ^{abc}	1.62 ± 0.11 ^{de}
GL	22.60 ± 0.34 ^{a}	85.07 ± 0.02 ^{d}	19.6 ± 0.2 ^{abc}	74.4 ± 0.6 ^{abc}	5.62 ± 0.35 ^{a}
CH	19.38 ± 0.33 ^{a}	86.02 ± 0.22 ^{cd}	16.0 ± 0.1 ^{d}	72.6 ± 0.2 ^{abc}	1.51 ± 0.04 ^{de}
NB	22.29 ± 1.84 ^{a}	81.64 ± 0.52 ^{f}	19.0 ± 0.3 ^{bc}	68.5 ± 1.1 ^{c}	1.45 ± 0.06 ^{de}
b)	*Proteins extracted using salt extraction-dialysis (SE)*				
YP	21.31 ± 1.03 ^{a}	86.93 ± 1.09 ^{bc}	18.9 ± 0.5 ^{bc}	76.8 ± 2.1 ^{a}	4.57 ± 0.51 ^{b}
GL	22.60 ± 0.34 ^{a}	85.16 ± 0.33 ^{d}	20.3 ± 0.4 ^{ab}	70.6 ± 1.4 ^{bc}	3.23 ± 0.31 ^{c}
CH	19.38 ± 0.33 ^{a}	87.73 ± 0.26 ^{b}	17.5 ± 2.1 ^{cd}	71.5 ± 4.7 ^{abc}	2.11 ± 0.60 ^{d}
NB	22.29 ± 1.84 ^{a}	83.29 ± 0.89 ^{e}	21.7 ± 0.7 ^{a}	75.9 ± 2.6 ^{ab}	1.47 ± 0.29 ^{e}

YP, yellow pea; GL, green lentil; CH, chickpea; NB, navy bean.

Means within a column followed by different letters indicate significant statistical differences ($p < 0.05$).

Extraction yields, representing the percentage of isolate obtained from an initial flour mass, ranged from ~ 16 to 22% and were affected by the extraction method ($p < 0.001$) and pulse ($p < 0.001$) but not their interaction. Overall, the extraction process using SE resulted in higher yields (~ 17–22%, d.b.) than those obtained using AEIP (~ 16–20%, d.b.) ($p < 0.001$). The type of pulse was also found to be significant, where NB and GL extraction yields (~ 20%) were found to be the highest followed by YP (~ 18%) and CH (~ 16%). Stone *et al.* (2015b) found that the SE method also resulted in higher extraction yields (~ 17–19%) from different pea cultivars compared to AEIP extractions (~ 15–16%).

Regarding protein yield, which represents the amount of protein recovered from the initial protein present in the flour, the AEIP extraction method resulted in protein yields of 68.5% for NB, 72.2% for YP, 72.6% for CH and 74.4% for GL. Meanwhile, the SE method resulted in 75.9% for NB, 76.8% for YP, 71.5% for CH and 70.6% for GL. The interaction between extraction method – pulse was found to be significant ($p < 0.001$). Specifically, the AEIP method yielded higher protein yields for CH and GL, whereas the SE method resulted in higher protein yields for YP and NB.

The phenolic content in the protein isolates obtained using different extraction methods was measured to explain differences found in the protein and extraction yields. Proteins and polyphenols can interact via reversible (hydrophobic interactions, hydrogen bonds and Van der Waals interactions) or irreversible (covalent bonds) interactions. These interactions can influence the proteins' structure, functionality (directly affecting solubility and solubility-related functional properties) and digestibility (Ozdal *et al.*, 2013). Overall, the SE samples were higher in TPC than those extracted using AEIP (Table 4.1). Sęczyk *et al.* (2019) studied protein phenolic interactions of white bean proteins, finding that for some phenolic compounds (*i.e.*, gallic acid and quercetin), the affinity for albumins was higher than for globulins. In contrast, some other phenolic compounds like catechins from green tea or chlorogenic acid from green coffee had higher affinity for the globulin fraction, so the affinity of phenolics for different protein fractions could have influenced the TPC levels, given that the AEIP method extracts mainly globulins, while the SE method also extracts albumins. The extraction method ($p < 0.01$), along with pulse type ($p < 0.001$) and their interaction, were found to be significant ($p < 0.001$) for the TPC in the samples. These differences in TPC in the extracted proteins using different extraction methods can explain why SE sample extraction yields were higher than for the AEIP extracted samples.

Regarding the pulse type, results from the present study showed that GL isolates had the highest phenolic content, followed by YP, CH and NB. A study conducted by Mondor *et al.* (2009), reported similar TPC contents for chickpea protein isolates obtained from defatted flours using the AEIP method. Furthermore, the authors observed differences in TPC when comparing AEIP with ultrafiltration/diafiltration (UF-DF) methods. Xu & Chang (2007) determined the TPC of different pulse flours using several extraction solvents different from the one used in this study (mixtures of acetone/water, acetone/water/acetic acid, absolute ethanol, ethanol–water and methanol–water). The phenolics found were in the range of those obtained in the present study for green lentil, pea

and bean. The authors also observed that the choice of solvent had an influence on the antioxidant activity and the composition of the extracted phenolics. Making a direct comparison of results with previous studies poses challenges due to variations in several factors, including the starting material (such as flour or protein concentrate/isolate), protein extraction method, phenolic extraction method (including the use of different solvents), and the determination method of phenolics (including the use of different standards for reporting TPC results). The variations among studies creates challenges when attempting to make straight-forward comparisons.

4.4.2. Physicochemical properties

4.4.2.1. Surface properties

Surface properties, such as surface charge and surface hydrophobicity of proteins, can indicate changes in the protein's surface due to extrinsic or intrinsic factors. These properties can provide information regarding protein interactions and therefore extend the understanding of their functionality. Zeta potential and surface hydrophobicity results are shown in Table 4.2. The surface charge showed all proteins had negative values at pH 7.0, where proteins carry a net negative charge. Zeta potential values ranged between − 32.0 to − 39.8 mV. Analysis of the zeta potential of the extracted samples showed that the extraction method ($p < 0.01$), type of pulse ($p < 0.001$) as well as their interaction ($p < 0.01$) had significant effects on the surface charge of the extracted proteins. Pulse proteins extracted through AEIP showed more negative zeta potential values than those obtained through SE. In the present study, a positive correlation emerged between zeta potential and albumin/globulin ratios. SE protein samples exhibited higher albumins than the AEIP samples, potentially explaining why the AEIP-extracted samples yielded a more negative zeta potential outcome than those obtained from SE.

Depending on the type of pulse used for extraction, differences were found due to the pulse source's intrinsic nature: zeta potential values ranged from – 32.4 to – 32.0 mV for NB, –32.8 to– 39.8 for YP, –38.8 to 38.3 for GL and –39.6 to – 38.8 for CH. Chang *et al.* (2022) and Stone *et al.* (2015) found lower zeta potential values for chickpea and pea-extracted proteins (~ − 25 and–21 mV, respectively), while Can Karaca *et al.* (2011c) found similar values ranging from – 35 to – 40 mV for lentil and chickpea, respectively. Keivaninahr *et al.* (2021) found similar zeta potential values for beans at ~ 34 mV.

Table 4.2. Surface properties and legumin/vicilin and albumin/globulin ratios of protein isolates at pH 7.0.

Pulse	Zeta potential (mV)	Surface Hydrophobicity (a.u.)	Legumin / Vicilin ratio	Albumin / Globulin ratio
a) Proteins extracted using alkaline extraction – isoelectric precipitation (AEIP)				
YP	-39.8 ± 1.8 ^{b}	6.0 ± 0.7 ^{cd}	0.74 ± 0.17 ^{c}	0.82 ± 0.18 ^{b}
GL	-38.8 ± 1.4 ^{b}	1.9 ± 0.2 ^{d}	0.76 ± 0.09 ^{c}	0.36 ± 0.10 ^{cd}
CH	-39.6 ± 1.7 ^{b}	5.7 ± 0.4 ^{b}	1.49 ± 0.26 ^{a}	0.13 ± 0.02 ^{d}
NB	-32.4 ± 2.1 ^{a}	16.1 ± 1.8 ^{a}	0.37 ± 0.09 ^{d}	0.42 ± 0.13 ^{cd}
b) Proteins extracted using salt extraction-dialysis (SE)				
YP	-32.8 ± 2.4 ^{a}	13.2 ± 1.1 ^{e}	0.77 ± 0.15 ^{c}	1.37 ± 0.29 ^{a}
GL	-38.3 ± 4.0 ^{b}	11.2 ± 1.0 ^{f}	0.93 ± 0.21 ^{bc}	0.53 ± 0.16 ^{cd}
CH	-38.8 ± 3.4 ^{b}	27.4 ± 2.3 ^{e}	1.21 ± 0.13 ^{ab}	0.29 ± 0.07 ^{bc}
NB	-32.0 ± 1.7 ^{a}	34.2 ± 3.5 ^{c}	0.27 ± 0.07 ^{d}	0.53 ± 0.16 ^{bc}

YP, yellow pea; GL, green lentil; CH, chickpea; NB, navy bean.

Means within a column followed by different letters indicate significant statistical differences ($p < 0.05$).

Surface hydrophobicity results indicate hydrophobic patches on the protein's surface, governed by the amino acid composition, protein conformation, and folding or unfolding levels present in the protein. A two-way analysis of the results showed that the pulse source ($p < 0.001$), and extraction method ($p < 0.001$) along with their interaction ($p < 0.001$) were found to be significant factors in surface hydrophobicity. Overall, samples extracted using the SE method had higher hydrophobicity than those obtained through AEIP. Stone *et al.* (2015b) found opposite results, where AEIP-extracted proteins showed higher hydrophobicity than those obtained through SE, explaining this could be because AEIP is more efficient at extracting globulins which have higher hydrophobicity compared to the SE method which also extracts albumins. In the present study, lower hydrophobicity in the AEIP samples could be due to possibly higher denaturation and unfolding of the proteins due to the extraction conditions (Krause *et al.*, 2002), which could lead to the interaction of proteins in solution forming small aggregates that overall, reduced the surface hydrophobicity. In line with these results, Osemwota *et al.* (2022) found that the extraction method, solvent of extraction, protein conformation and amino acid content, can influence hydrophobicity, supporting the observed differences in surface hydrophobicity.

4.4.2.2. Legumin/vicilin (L/V)–albumin/globulin (A/G) ratios

Determination of L/V and A/G ratios are reported in Table 4.2. The major bands found for pulses were legumin (~ 40 and ~ 20 kDa), vicilin (~ 50 and ~ 30 kDa), convicilin (~ 71 kDa) and albumins (< 15 kDa). The L/V ratios found for pulse protein concentrates extracted with AEIP and SE methods showed that the type of pulse source ($p < 0.001$) and the interaction of extraction method and pulse type were found to be significant factors ($p < 0.05$). The L/V ratios were higher for AEIP CH (~ 1.49), followed by SE CH > SE GL > SE YP > AEIP GL > AEIP YP > AEIP NB, and lowest for SE NB (~ 0.27). The L/V ratios positively correlated with protein content ($p < 0.05$, r = 0.3152). Mertens *et al.* (2012) also found a positive correlation and positive relationship between L/V ratios and protein content in smooth peas. The ratios found in the present study are similar to those reported for these pulses in previous studies for navy beans (Guldiken *et al.*, 2021), peas and lentils (Swanson, 1990) and chickpeas (J. Boye *et al.*, 2010). However, the L/V ratios in pulses can be affected by different intrinsic (type of pulse and cultivar) and extrinsic factors (agronomical factors such as location, environmental conditions and harvest) (Mertens *et al.*, 2012).

The A/G ratios were also determined (Table 4.2), and these ratios were higher for the samples that were prepared using the SE method ($p < 0.001$). This suggests that extraction under alkaline conditions followed by precipitation of proteins at the isoelectric point achieved precipitation of mainly globulins (pI ~ 4.5), while albumins remained in the supernatant (pI ~ 6) (Swanson, 1990) and were discarded. On the contrary, SE-extracted proteins achieved higher albumin extraction since there was no isoelectric point precipitation step that selected mainly one type of protein, but rather the salt-extracted proteins were freeze-dried after being dialyzed, keeping both albumins and globulins in solution. Similar results were found by Tanger *et al.* (2020), where no albumins were found on AEIP-extracted proteins compared to proteins extracted with the SE method. However, no A/G ratios were determined for further ratio comparison on the SE samples. In terms of the different pulses evaluated, the type of pulse was found to be significant for the A/G ratios found ($p < 0.001$), and they were found in the following order: YP > NB = GL > CH. The interaction pulse and extraction method was also significant ($p < 0.001$).

4.4.3. Functional properties

4.4.3.1. Solubility

Table 4.3 presents the results of percent protein solubility of the extracted proteins at pH 7.0. Analysis of results showed that the extraction method ($p < 0.001$), the type of pulse ($p < 0.001$) and their interaction were found significant ($p < 0.001$) in the solubility of the protein isolates. For YP, GL and CH, the solubility of the resulting proteins extracted via the SE method was higher than proteins obtained using AEIP, except for NB, which had the opposite result. The highest solubility was found for SE-GL proteins (~ 91%), followed by AEIP NB (~ 87%), while the lowest was reported for AEIP YP and GL (66.30 and 57.59%, respectively). Stone *et al.* (2015b) found higher solubilities for SE-extracted pea proteins than those extracted using AEIP or micellar precipitation (MP). The authors attributed these differences in solubilities due to hydrophobic interactions between proteins. However, in the present study, the hydrophobicity resulted in higher values for the SE samples than the AEIP-extracted ones. Can Karaca *et al.* (2011a) studied AEIP and SE extracted proteins on different pulses and suggested that differences in the solubility were reflected due to differences in the protein composition of the extracted proteins. Mundi & Aluko (2012) attributed the higher solubility of albumins in kidney bean proteins to higher glycation of this fraction, leading to more interactions of carbohydrates with water and increased solubility compared to the globulin fraction. In the present study, the SE extracted samples resulted in higher A/G ratios, which could explain higher solubilities than the AEIP samples, due to higher albumins in these samples. In the case of NB, which exhibited higher solubilities for the AEIP extracted proteins despite their lower A/G ratios, could be due to the higher hydrophobicity of the SE samples that could have impacted the aggregation and folding of these proteins, leading to decreased solubility. No correlations between solubility and zeta potential or surface hydrophobicity were found.

Table 4.3. Functional properties of protein isolates at pH 7.0

Pulse	Solubility (%)	EAI (m2/g)	ESI (min)	FC%	FS%	WHC (g/g)	OHC (g/g)
a) Proteins extracted using alkaline extraction – isoelectric precipitation (AEIP)							
YP	66.30 ± 3.56 ^{d}	23.68 ± 1.55 ^{c}	12.36 ± 0.97 ^{ab}	247.8 ± 11.5 ^{d}	89.2 ± 3.6 ^{a}	1.72 ± 0.02 ^{b}	1.27 ± 0.07 ^{d}
GL	57.59 ± 3.07 ^{e}	26.91 ± 1.73 ^{b}	11.90 ± 0.50 ^{ab}	220.0 ± 11.2 ^{e}	87.7 ± 3.8 ^{a}	1.34 ± 0.09 ^{c}	0.64 ± 0.09 ^{f}
CH	74.85 ± 3.01 ^{bc}	28.12 ± 1.00 ^{ab}	12.58 ± 0.46 ^{a}	231.1 ± 10.0 ^{de}	87.5 ± 1.2 ^{a}	1.69 ± 0.08 ^{b}	1.05 ± 0.06 ^{e}
NB	87.85 ± 3.39 ^{a}	29.01 ± 0.54 ^{ab}	11.94 ± 0.33 ^{ab}	221.1 ± 9.8 ^{e}	87.9 ± 3.5 ^{a}	1.69 ± 0.05 ^{b}	0.79 ± 0.09 ^{f}
b) Proteins extracted using salt extraction-dialysis (SE)							
YP	71.09 ± 4.23 ^{cd}	30.08 ± 1.54 ^{a}	12.44 ± 0.25 ^{ab}	283.3 ± 11.0 ^{c}	84.3 ± 4.8 ^{a}	0.93 ± 0.07 ^{d}	1.55 ± 0.07 ^{c}
GL	91.05 ± 3.03 ^{a}	29.90 ± 0.68 ^{a}	12.28 ± 0.51 ^{ab}	312.2 ± 9.8 ^{b}	84.3 ± 3.2 ^{a}	1.91 ± 0.16 ^{a}	1.88 ± 0.17 ^{b}
CH	80.55 ± 3.23 ^{b}	29.02 ± 1.72 ^{ab}	11.47 ± 0.63 ^{b}	342.2 ± 13.8 ^{a}	82.1 ± 2.8 ^{a}	0.95 ± 0.07 ^{d}	2.48 ± 0.10 ^{a}
NB	60.07 ± 3.57 ^{e}	26.89 ± 1.16 ^{b}	11.57 ± 0.67 ^{ab}	317.8 ± 13.1 ^{b}	68.5 ± 6.1 ^{a}	1.39 ± 0.11 ^{c}	2.02 ± 0.13 ^{b}

YP, yellow pea; GL, green lentil; CH, chickpea; NB, navy bean; EAI, emulsion activity index; ESI, emulsion stability index; FC, foaming capacity; FS, foaming stability; WHC, water hydration capacity; OHC, oil holding capacity.

Means within a column followed by different letters indicate significant statistical differences ($p < 0.05$).

4.4.4. Emulsion activity and stability index (EAI–ESI)

Emulsion activity and stability were investigated at pH 7.0 using canola oil (Table 4.3). A two-way analysis of variance showed that pulse ($p < 0.05$), as well as the extraction method ($p < 0.001$) and their interaction ($p < 0.001$), were found to be significant factors for EAI. Overall, emulsions prepared from SE were more effective at emulsion formation, showing higher EAI than the AEIP proteins. Emulsion activity indexes were similar for SE YP, GL and CH (30.08, 29.90 and 29.02 m^2/g, respectively, $p > 0.05$), while NB presented the lowest EAI value (26.89 m^2/g). Emulsions for the AEIP proteins ranged from 23.68 to 29.01 m^2/g. The improved emulsifying activity from SE- prepared isolates can be explained by the higher solubility of the SE proteins compared to the AEIP isolates. Emulsifying properties are influenced by the protein's ability to solubilize since it is a prerequisite for the protein to migrate and accommodate at the interface and reduce the interfacial tension (Can Karaca *et al.*, 2011a). In the present results, EAI correlated positively with solubility (r = 0.50, $p < 0.05$). Can Karaca *et al.* (2011a). found higher EAI values for canola proteins extracted using a salt extraction method compared to alkaline extraction, while no differences were found in EAI for the method of extraction in flaxseed proteins. However, Can Karaca *et al.* (2011b) found the opposite results when determining EAI for pulse proteins (chickpea, faba bean lentil and pea). The SE samples resulted in higher emulsifying properties than the proteins obtained through the AEIP method. This was attributed to the higher surface charge and solubility of the proteins, leading to higher EAI.

Emulsion stability showed a significant interaction between the method of extraction and pulse ($p < 0.05$). YP and GL SE isolates had higher stabilities than their AEIP counterparts (12.44 and 12.28 min, versus 12.36 and 11.90 min, respectively). At the same time, NB and CH showed AEIP concentrates to have higher ESI (12.58- NB and 11.94-CH for the AEIP method, versus 11.47 and 11.57 min for the SE method). This property negatively correlated with surface hydrophobicity (r = – 0.41, $p < 0.05$). This could be explained given that when proteins with more hydrophobic groups surround droplets, hydrophobic interactions are favoured in neighbouring proteins, bringing oil droplets closer together and promoting emulsion instability (Lam & Nickerson, 2013). ESI values for the SE samples were similar to those reported for pulses (chickpea, faba bean, lentil and pea) by Can Karaca *et al.* (2011b), however AEIP values found by the authors were higher than the ones determined in the present study. Differences between results of the present study and previous research regarding emulsifying properties can be attributed to variations in the

emulsification method and conditions employed. Factors such as homogenization time, speed, and equipment used can impact the resulting emulsifying properties of the proteins (McClements *et al.*, 2022).

4.4.4.1. Foaming capacity and stability (FC–FS)

Foaming results at pH 7.0 are shown in Table 4.3. The legume source ($p < 0.001$), extraction method ($p < 0.001$) as well as their interaction ($p < 0.001$) was found to be significant in the foaming capacity of the extracted proteins. Overall, FC was significantly higher for the SE samples than AEIP-extracted proteins, with values ranging from ~ 283 to 342% for foams prepared with SE-extracted proteins and values ranging from ~ 220 to 247% for the foams produced with AEIP-extracted proteins. Regarding the type of pulse, CH-extracted proteins had the highest foaming capacity, followed by NB, GL and YP. In the present study, FC was positively correlated with surface hydrophobicity ($p < 0.05$, r = 0.71). SE extracted samples showed higher surface hydrophobicity values than AEIP and higher FC, suggesting that the higher the hydrophobic groups available, the greater their interaction at the interface and formation of a film surrounding the air bubbles. Surface hydrophobicity can facilitate the initial accommodation of hydrophobic groups to the interface. However, FC was reported to positively correlate with the measurement of average surface hydrophobicity, since it takes into account not only the hydrophobic interactions but also the interactions and properties of unfolded proteins at the interface (Damodaran, 2006). Ghumman *et al.* (2016) found that the albumin fraction exhibited higher FC compared to the globulin fraction for lentils and horse gram proteins, hypothesizing that this difference could be attributed to globulins undergoing less unfolding due to their more compact structure at the interface than albumins. The higher FC values obtained by the proteins extracted through the SE method in our study further support this hypothesis. We observed higher A/G ratios for the SE-extracted proteins compared to the ratios determined through SDS-PAGE analysis for the AEIP ratios (Table 4.2).

The analysis of FS, using a two-way ANOVA revealed a significant effect of the extraction method ($p < 0.001$). Notably, FS of AEIP pulse proteins (ranging from 87.5 to 89.2%) was found to be higher than SE proteins (68.5 to 84.3%). These results indicate that the choice of extraction method significantly influences the foam stability of the proteins, with AEIP demonstrating superior performance in this functional property. The pulse type nor the interaction pulse and extraction method were not significant factors for FS. Results are shown in Table 4.3. Stone *et al.*

(2015b) found that the extraction method was a significant factor in FS for pea proteins; however, they reported that the AEIP isolates resulted in similar FS for different pea cultivars, whereas proteins extracted through the SE or MP methods resulted in variable results depending on the cultivar. The higher A/G ratio observed in the SE-extracted proteins provides a possible explanation for their lower FC compared to those extracted through AEIP. This finding aligns with the study conducted by Mundi & Aluko (2012) where they evaluated the functionality of extracted globulins and albumins fractions of kidney bean. In this study, they found that albumins resulted in lower FS than globulins. The authors attributed this result to the albumins interacting with sugars, leading to lower surface hydrophobicity of the proteins that could therefore decrease protein–protein interactions at the interface and create instability in maintaining a strong viscoelastic film surrounding the air bubbles. In the present study, however, the hydrophobicity of the SE samples, which resulted in lower FS, was higher than those extracted by AEIP. In the present study, we observed a positive correlation between FS with protein content ($p < 0.05$, r = 0.30). These findings indicate that the higher protein content contributes to the formation of a viscoelastic film around the air bubbles improving the foam stability. This aligns with previous research by Ma *et al.* (2022a) which supports the notion that increased protein concentration leads to improved FS.

4.4.4.2. Water and oil holding capacity (WHC–OHC)

WHC and OHC, defined as the amount of water or oil that 1 g of protein can retain, are shown in Table 4.3. Samples extracted via AEIP were found to have higher WHC than the samples obtained through salt extraction ($p < 0.001$). The pulse source, as well as the interaction pulse and extraction method, were also found to be significant ($p < 0.001$). Overall, GL and NB were the proteins that showed more hydration capacity than CH or YP. WHC values were found to negatively correlate with TPC (– 0.36, $p < 0.05$) and with surface hydrophobicity (– 0.37, $p < 0.05$). The presence of hydrophobic groups on the protein's surface can contribute to a reduction in protein-water interactions, leading to decreased water retention. This observation gives a potential explanation for the lower hydration capacity observed in the SE samples, which exhibited higher surface hydrophobicity compared to the AEIP samples. The increased surface hydrophobicity of the SE samples may hinder the protein's ability to bind and retain water, resulting in lower WHC. Martinez *et al.* (2016) found lower WHC for different cultivars of faba bean protein concentrates extracted through air classification than the navy beans WHC found in

this study. Stone *et al.* (2015b) found similar WHC results ranging from 1.91 to 2.37 g/g for different pea cultivars extracted using an alkaline extraction and acid precipitation method. These differences can be due to the protein extraction method, structure, and the proteins' groups readily available to interact with water.

Oil holding capacities analysis also showed that the extraction method ($p < 0.01$), pulse ($p < 0.01$) and their interaction were found to be significant ($p < 0.01$). OHC results were opposite to those obtained for WHC, where in this case, SE samples were found to have higher OHC (1.55 to 2.48 g/g) than the AEIP samples (0.64 to 1.27 g/g). Osemwota *et al.* (2022) studied the OHC of lentil protein fractions and found that the albumins presented higher oil-holding capacities than the globulin fraction, possibly due to a more folded structure of the globulin proteins, which led to less interaction of the hydrophobic groups with oil. Mundi & Aluko (2012) found similar results for albumin and globulin protein fractions extracted from kidney beans. In this study, the SE samples, which presented higher OHC, also showed higher albumin to globulin ratios, which could explain the improved capacity of the SE samples to retain oil.

Stone *et al.* (2015b) also found that SE pea protein samples presented higher OHC compared to those extracted by AEIP. However, OHC values found in the present study were lower than those reported by the authors (5.2–5.4 g/g for SE proteins and 3.5–3.8 g/g for AEIP samples). In a study conducted by Boye *et al.* (2010) OHC values for chickpea, bean and pea fell within a similar range as the values observed in this study. On the contrary, Shi & Nickerson (2022) reported higher OHC values (~ 1.63 g/g) for faba bean proteins extracted using AEIP compared to the AEIP-extracted navy beans in our study. A positive correlation (0.72, $p < 0.05$) was observed between OHC and surface hydrophobicity. This finding suggests that proteins with higher levels of hydrophobic moieties on the surface of the protein facilitate greater interactions via hydrophobic interactions with the oil, resulting in the enhanced ability of the protein to retain oil. In our study, a negative correlation ($p < 0.05$, $r = -0.35$) was found for the WHC and OHC. These findings align with those found by Toews & Wang (2013) who studied the functionality of peas, lentils, chickpeas and navy beans protein concentrates obtained by an alkaline extraction and acid precipitation of the proteins. These similar results suggest that there is a compromise between the ability of the proteins to retain oil and water.

4.4.5. Protein quality

4.4.5.1. Amino acid composition

Table 4.4 displays the results for protein quality. Across all the examined pulses, methionine, cysteine, and tryptophan were the amino acids present at lower levels. In contrast, aspartic and glutamic acid were found to be the most abundant, followed by leucine and arginine. It is worth noting that pulses are commonly characterized by the presence of these amino acids as limiting (Boye *et al.*, 2010). Previous studies reported similar results for pea, navy bean and chickpea (Nosworthy *et al.*, 2017; Nosworthy & House, 2017). In the present study, both extraction methods yielded navy bean samples with methionine and cysteine as the limiting amino acids. This finding is consistent with previous research by Nosworthy *et al.* (2018) which reported that sulphur-containing amino acids were limiting for extruded, cooked and baked navy beans. Similarly, Guldiken *et al.* (2021) found similar results for navy bean flours. For YP, GL and CH, the limiting amino acid was tryptophan, followed by cysteine and methionine. Similar results were found by Nosworthy *et al.* (2017) for cooked chickpeas and split yellow peas, while green lentil was found limiting in methionine and cysteine. The limiting amino acid scores reported in previous studies for lentil protein isolate and concentrate (0.52 and 0.75, respectively), pea protein isolates and concentrate (0.54 and 0.58, respectively) (Nosworthy & House, 2017) and pea protein enriched flour (0.79) (Konieczny *et al.*, 2020), where found to be comparable to the results obtained in this study. The scores for limiting amino acids in chickpeas (0.71) were observed to be lower than those reported by Stone *et al.* (2019) for desi and kabuli chickpeas (ranging from 0.96 to 1.04). Similarly, Guldiken *et al.* (2021) reported amino acid scores for navy bean flours of different varieties to range between 0.79 and 1.03, with our study yielding a score of 0.72. These comparisons highlight the variations among pulse varieties, reported by Nosworthy & House (2017).

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

Results for *in-vitro* protein digestibility (IVPD) are shown in Table 4.4. An analysis of variance showed that both the extraction method ($p < 0.001$) and pulse source were significant factors for IVPD ($p < 0.001$), but not their interaction. Regarding the extraction method, alkaline extracted samples showed higher IVPD values than those extracted using SE. This could be attributed to overall higher phenolic contents in the SE samples, suggesting that the higher presence of phenolic

compounds interacting with proteins can result in a decrease in their bioavailability (Mondor *et al.*, 2009). Additionally, our findings revealed a negative correlation between IVPD and TPC ($p < 0.05$, r = -0.45). Research conducted by Stone *et al.* (2015b) and Tanger *et al.* (2020) suggests that proteins extracted by the AEIP method undergo greater denaturation compared to those extracted using SE. This can be attributed to the fact that AEIP extraction conditions are known to be harsh. Consequently, this could lead to protein unfolding and more accessible access of the enzymes to cleave peptide bonds in the AEIP samples. In our study, a significant positive correlation ($p < 0.05$, r = 0.67) was found between IVPD and protein content. This finding aligns with the research conducted by Park *et al.* (2010), who also reported a positive correlation. These results reinforce the fact that higher protein content contributes to enhanced protein digestibility.

Table 4.4. Amino acid scores, *in-vitro* protein digestibility values (IVPD) and *in-vitro* protein digestibility corrected amino acid score (IVPDCAAS) of protein isolates.

Pulse / Extraction method	Limiting amino acid	Amino-acid score	*In-vitro* protein digestibility - IVPD (%)	*In-vitro* protein digestibility corrected amino-acid score - IVPDCAAS (%)
a) Proteins extracted using alkaline extraction – isoelectric precipitation (AEIP)				
YP	TRP	0.74	86.99 ± 0.80 ^{a}	63.94 ± 0.59 ^{a}
GL	TRP	0.54	84.30 ± 1.57 ^{b}	45.61 ± 0.85 ^{e}
CH	TRP	0.71	81.44 ± 1.08 ^{cd}	57.70 ± 0.76 ^{c}
NB	MET + CYS	0.72	82.34 ± 0.35 ^{bc}	59.32 ± 0.25 ^{b}
b) Proteins extracted using salt extraction-dialysis (SE)				
YP	TRP	0.80	80.02 ± 1.08 ^{de}	64.08 ± 0.86 ^{a}
GL	TRP	0.56	78.42 ± 1.47 ^{e}	43.64 ± 0.82 ^{f}
CH	TRP	0.72	76.16 ± 0.94 ^{f}	54.82 ± 0.68 ^{d}
NB	MET + CYS	0.80	75.16 ± 1.02 ^{f}	60.33 ± 0.82 ^{b}

YP, yellow pea; GL, green lentil; CH, chickpea; NB, navy bean; MET, methionine; CYS, cysteine; TRP, tryptophan. Means within a column followed by different letters indicate significant statistical differences ($p < 0.05$).

The albumin fraction of pulse proteins has been found to have lower IVPD than those determined in the globulin fractions. Genovese & Lajolo (1996) studied the digestibility of albumins and found that the low digestibility of these proteins can be due to different factors, such as glycosylation of albumins that produce a steric impediment for the enzymes to cleave bonds, more rigid structures due to the presence of disulphide bonds, as well as the presence of protease inhibitors. Certain protease inhibitors, including lectins and lipoxygenases, are known to be albumins, and their presence can impact protein digestibility (Park *et al.*, 2010). Consistent with this information, the SE samples exhibited higher A/G ratios (shown in Table 4.2), which could explain their lower digestibility.

IV-PDCAAS was determined by multiplying IVPD and the limiting amino acid score, determined by the limiting amino acid in the sample and the FAO scores. IV-PDCAAS gives an indication of protein quality, and it considers not only the *in-vitro* digestibility but also the limiting amino acids in the sample. As mentioned in the amino acid composition, the common limiting amino acids for pulses are the sulphur-containing amino acids and tryptophan. Methionine and cysteine were found to be limiting for the NB samples, while tryptophan was found to be limiting for YP, GL and CH protein isolates, for both extraction methods. The corrected amino-acid scores analysis showed that the extraction method ($p < 0.001$), pulse ($p < 0.001$) and their interaction ($p < 0.001$) had a significant effect on IV-PDCAAS. Results are shown in Appendix A. Like found for IVPD results, the samples obtained by alkaline extraction—isoelectric precipitation showed higher IVP- DCAAS than those extracted by salt extraction-dialysis, in the following order: YP AEIP = YP SE > NB SE = NB AEIP > CH AEIP > CH SE > GL AEIP > GL SE.

4.5. Conclusions

This study explored the impact of different extraction methods on the yields, physicochemical, functional and nutritional properties of protein isolates derived from different pulses. The findings revealed that the extraction method and pulse type did not consistently emerge as significant factors affecting protein extraction yield or protein content of the isolates. However, the choice of extraction method noticeably influenced the surface properties and functional attributes such as solubility, emulsifying, foaming, and water and oil holding capacities. In general, SE-prepared isolates exhibited higher solubility, FC and EAI compared to those obtained through the AEIP method. On the other hand, with regards to protein quality, AEIP-prepared isolates showed higher

IVPD and IV-PDCAAS than the SE samples. Furthermore, the pulse source also influenced the protein content and functionality of the isolates, highlighting the importance of pulse variety in assessing pulse protein characteristics and functionality, directly impacting the application where these proteins will work better. In consequence, these results suggest that if a specific application of the protein is desired, choosing a specific pulse variety could show superior performance compared to alternatives, concluding that pulse type has a direct impact on their target use.

4.6. Connection to the next study

This study explored the effect of using two different extraction methods on the functional and nutritional properties of the protein derived from four pulse crops. Results showed that the extraction method and the type of pulse were influential on the resulting functional properties of the isolates as well as their nutritional properties. Therefore, careful selection of these parameters can impact the use and application of the proteins. The use of plant proteins is sometimes limited by their functionality. Low solubility, for example, can impact other functional properties such as emulsifying and foaming properties. To improve plant protein functionality and evaluate how proteins can be tailored to perform as good foaming and emulsifying agents, the following study will explore the effect of pH shifting (acidic and alkaline) in combination with heat on a commercial pea protein isolate. The goal of the next study is to modify the protein surface properties to improve their functionality.

5. THE EFFECT OF HEAT AND PH SHIFTING ON A COMMERCIAL PEA PROTEIN ISOLATE

5.1. Abstract

In the present study, the effect of a pH shifting method with or without heating was investigated for its effects on the resulting surface and functional properties of a commercial pea protein isolate. This method involves the use of acid and base to change the pH of a protein solution to promote structure modifications by inducing protein unfolding and refolding. Half the samples were subject to pH shifting at both acidic (pH 2), neutral (pH 7) and alkaline (pH 10) pH at room temperature, while the heat-treated samples followed a 1h heating at 80ºC. All the samples were adjusted back to neutrality, and the surface and functional properties were determined at pH 7. The results of these treatments indicated that an alkaline pH shifting, as well as its combination with heat, resulted in a significant increase in the solubility of the proteins, whereas an acidic pH shifting, as well as in combination with heat, reduced the stability of the proteins in solution. Additionally, some functional properties were enhanced by alkaline pH shifting, such as foaming capacity or emulsion stability; however, other properties showed no alteration or were negatively impacted by alkaline and acidic pH shifting, such as foaming stability. Analysis of the bubble structure of foams prepared using a sparging method revealed that that bubble sizes for samples shifted at pH 2 in combination with heating presented the biggest increase in bubble growth over time, creating a less stable foam. The application of a pH shift treatment and the use of heat can aid in the improvement of pea protein functionality and allow the tailoring of these proteins for applications in food products.

5.2. Introduction

With the increasing demand for ingredients from plant proteins, pulses, such as peas, chickpeas and beans, have been fulfilling the growing demands since their protein contents range between ~20%-32%. Extracted proteins from plant sources, however, tend to have a lower performance in their functionality compared to animal-source proteins. This difference can be mainly attributed to the structure difference between these types of proteins. While animal source proteins tend to be

more flexible allowing their accommodation to interfaces, which translates to good emulsifying or foaming properties for example, plant sources tend to be globular and more rigid in their structure, hindering their interaction with other proteins, as well as presenting lower solubility, which tends to limit their application in food products (McClements & Grossmann, 2021).

To address this functionality shortcoming, plant proteins can be subject to different processes to modify their structure leading to an improvement in their functional properties. Some examples of these protein modification strategies are physical modifications such as heat treatments or extrusion; biological methods such as enzymatic modification or fermentation, and chemical modifications (Nasrabadi *et al.*, 2021). Among these latter strategies, pH shifting is a method that can improve the solubility, foaming, emulsifying and gelling properties of plant proteins such as faba bean, pea, chickpeas, rice, oats and hemp (Alavi *et al.*, 2021; Chang *et al.*, 2023; Tang *et al.*, 2023; Wang *et al.*, 2022). Protein changes driven by pH shifting at alkaline or acidic pH occur when groups with the same charge repel one another promoting protein unfolding, followed by protein re-folding when the pH is adjusted back to neutrality. Protein unfolding can, therefore, expose previously buried hydrophobic and functional groups towards the surface of the protein and provide the protein with more surface activity that translates to heightened functionality (Jiang *et al.*, 2018). The pH shifting method has also been combined with other physical methods, such as ultrasound, heating, crosslinking, and cold plasma with the objective of improving their emulsifying, foaming, and gelling properties (Chang *et al.*, 2023; Sun *et al.*, 2023; Zhang *et al.*, 2022; Zhao *et al.*, 2023). This study aims to evaluate the effect of pH shifting and heat on the behaviour of pea protein emulsions and foams. This study examined two pH conditions: acidic (pH 2) and alkaline (pH 10) as well as the effect of temperature (heating at 80ºC). Emulsifying properties were studied by preparing coarse emulsions using a high-speed homogenizer, and their droplet sizes were determined. Foaming properties were prepared using a high-speed homogenizer as well as using a novel dynamic foam analyzer (DFA) that allowed the exploration of the foam structure, contributing to a more detailed study of the foam stability. The findings of this study will contribute and add to the understanding of protein behaviour, as well as provide insights for the application of pea proteins into food products as emulsifying and foaming agents such as in beverage applications, whipped preparations, or aerated products.

5.3. Materials and methods

5.3.1. Preparation of pH-shifted and heated pea protein samples.

A commercial pea protein isolate was obtained from AGT Foods Research and Innovation centre (Saskatoon, SK, Canada) and produced through an alkaline extraction-isoelectric precipitation process (80.1% protein, dry weight basis). The pH-shifted samples were prepared according to the method by Tang *et al.* (2023) with some modifications. Protein solutions of 10% (w/w) concentration (based on protein content) were prepared using Milli-Q® water (Millipore Corporation) and stirred overnight at 4ºC to promote solubilization. The following day, the samples were stirred for 30 min at room temperature. Afterwards, the pH of the solution was adjusted to 2, 7 or 10 using 1 N HCl or NaOH and stirred for 1 h. Half the samples were kept at room temperature and adjusted back to pH 7 using 1N HCl or NaOH and stirred for 1h. Hereafter these samples will be referred to as pH_2, pH_7 and pH_{10}. The other samples were heat-treated; therefore, after stirring for 1h at the corresponding pH, they were placed in a shaking water bath at 80ºC for 1 h (VWR International, Mississauga, ON, Canada). Afterwards, they were cooled to room temperature and the pH was adjusted back to pH 7 using 1 N HCl or NaOH. Hereafter the heat-treated samples will be referred to as pH_{2heat}, pH_{7heat} and pH_{10heat}. All the samples were adjusted to 10% (w/w) protein after sample preparation to account for the addition of acid and base during pH adjustment as well as evaporation during the heating or stirring step.

5.3.2. Surface properties

5.3.2.1. Zeta potential

Solutions of 0.05% (w/w) concentration were prepared and stirred at pH 7 for 1 h. A Zetasizer Nano-ZS90 (Malvern Instruments, Westborough, MA, USA) was used to measure the surface charge of the samples. The electrophoretic mobility was determined (U_E) and the zeta potential (ζ) was calculated using Henry's equation:

$$U_E = \frac{2\varepsilon\,\zeta\,f(\kappa\alpha)}{3\eta} \qquad \text{(Eq.5.1)}$$

Where ε represents the permittivity, $f(\kappa\alpha)$ is a function of the Debye length and the particle radius, and where η represents the viscosity.

5.3.2.2. Surface hydrophobicity

Surface hydrophobicity was determined using the ANS-probe method with some modifications (Kato & Nakai, 1980; Li-Chan *et al.*, 1985). Solutions of 0.025% (w/w) concentration were prepared and stirred at pH 7 for 1 h. Successive dilutions of 0.005, 0.010, 0.015, 0.020 and 0.025% concentration were prepared from the initial solution. Afterwards, 1.6 mL was taken from each solution and 0.20 µL of ANS was added, vortexed and kept in the dark for 5 min to allow the reaction to occur. Measurements were performed on a Fluoromax-4 fluorometer (Horiba Jobin Yvon Inc., Edison, NJ, USA) using 390 nm excitation and 470 nm emission wavelengths with slits of 1 nm bandpass. Protein blanks with the addition of 0.20 µL of water were also run and their measurements were subtracted from the protein solutions. Surface hydrophobicity was determined as the slope of concentration vs. intensity.

5.3.3. Functional properties

5.3.3.1. Solubility

Protein solubility was determined using the method followed by Morr *et al.* (1985) and Tang *et al.* (2023) with some modifications. Protein solutions (1% w/w concentration) were prepared from the pH-shifted solutions and stirred for 1 h at pH 7.0. The solutions were then transferred to a 15 mL centrifuge tube and centrifuged at 4180 g for 10 min (Thermo Scientific Sorvall ST-8, Waltham, MA, USA). The supernatant was collected, and the protein content was determined using a micro-Kjeldahl digestion and distillation unit (Labconco Co., Kansas City, MO, USA). A factor of 6.25 was used to convert %Nitrogen to protein. Protein solubility was calculated as the percentage of the protein present in the supernatant divided by the protein content of the initial sample.

5.3.3.2. Emulsifying properties

5.3.3.2.1. Emulsion Activity (EAI) and Stability Indices (ESI)

Emulsion activity and stability indexes were determined following the method by Pearce & Kinsella (1978). Protein solutions of 0.25% (w/w) concentration were prepared and stirred for 1 h at pH 7. Emulsions were prepared by homogenizing in 50 mL centrifuge tubes: 5 g of the protein solutions and 4 g of canola oil using an IKA T-10 Basic ULTRA-TURRAX Homogenizer (IKA Works, Wilmington, NC, USA) for 5 min at speed 5 (13000 rpm). Immediately after

homogenization and after 10 min, an aliquot of 50 µL was taken from the bottom of the tube and added to 7.5 mL of a 0.1% SDS solution followed by vortexing for 10 s. The absorbance was measured at 500 nm using 1 cm path plastic cuvettes on a Genesys 20 UV-vis spectrophotometer (Thermo Fisher Scientific). EAI and ESI were calculated as follows:

$$EAI\ (\frac{m^2}{g}) = \frac{2 \times 2.203 \times A_0 \times N}{c \times \varphi \times 10000} \quad \text{(Eq. 5.2)}$$

$$ESI\ (\text{min}) = \frac{A_0}{\Delta A} \times t \quad \text{(Eq. 5.3)}$$

Where A_0 represents the intial absorbance, N represents the dilution factor (f=150), c is the weight of the protein per mL, φ is the volume fraction of the oil, t represents time (t=10 min) and ΔA is the change in absorbance taken at time cero and after 10 min. EAI represents the interfacial area stabilized by g of protein and ESI represents the stability of the emulsion over time.

5.3.3.2.2. Emulsion Stability (ES)

Emulsion stability was determined using the method based on creaming reported by Can Karaca *et al.* (2011b). The method was determined by preparing emulsions using 5 g of canola oil and 5 g of a 1% (w/w) protein solution. The protein solutions were adjusted to pH 7.0 and stirred for 1h before preparing the emulsions. An IKA T-10 basic ULTRA-TURRAX Homogenizer (IKA Works, Wilmington, NC, USA) was used to homogenize the samples for 5 min at speed 5 (13000 rpm). Immediately after homogenization, the emulsions were transferred to 10 mL graduated cylinders. The drainage of a serum layer was determined after 1 h the stability of the emulsion was calculated as follows:

$$\%\ ES = \frac{V_a - V_b}{V_a} \times\ 100 \quad \text{(Eq. 5.4)}$$

Where Va represents the volume of the aqueous phase prior to emulsification, and V_b represents the serum layer separated from the emulsion after drainage.

5.3.3.2.3. Emulsion droplet size

A Mastersizer (Mastersizer 2000, Malvern Instruments Ltd.) was used to measure the particle size distribution of the emulsion droplets following the method reported by Wang *et al.* (2022). An IKA T-10 Basic ULTRA-TURRAX Homogenizer (IKA Works, Wilmington, NC, USA) was used to prepare the emulsions using 5 g of canola oil and 5 g of a 1% (w/w) protein solution adjusted at pH 7 and stirred for 1 h. The samples were homogenized for 5 min at speed 5 (13000 rpm). Immediately after the emulsion preparation, 4 droplets of emulsion were added to the sample cell for determination at a ~14% obscuration. The particle size distribution is reported as the surface-weighted average diameter (D_{32}) represented as follows:

$$D_{3,2} = \frac{\sum_{i=1} N_i d_i^3}{\sum_{i=1} N_i d_i^2} \quad \text{(Eq. 5.5)}$$

Where N_i represents the number of droplets of a d_i diameter (McClements, 2015).

5.3.3.3. Foaming properties

5.3.3.3.1. Foaming capacity and stability

Foaming capacity (FC) and foaming stability (FS) were determined following the method by Liu *et al.* (2010) with some modifications. In summary, 15 mL (V_l) of 1% (w/w) protein solutions at pH 7 were transferred to 400 mL glass beakers and homogenized using an IKA T-10 Basic ULTRA-TURRAX Homogenizer (IKA Works, Wilmington, NC, USA). The samples were homogenized for 5 min at speed 5 (13000 rpm) and transferred to 100 mL graduated cylinders immediately after homogenization. The initial volume (V_0) of the foam and the volume of the foam after 30 min (V_f) were recorded and FC and FS were calculated as follows:

$$\%FC = \frac{V_0}{V_l} \times 100 \quad \text{(Eq. 5.6)}$$

$$\%FS = \frac{V_f}{V_0} \times 100 \quad \text{(Eq. 5.7)}$$

5.3.3.3.2. Foam structure analysis

The foam structure of all samples was determined using a Dynamic Foam Analyzer (DFA) 100 (KRUSS, Hamburg, Germany) following the method by Oetjen *et al.* (2014) with modifications.

In brief, 50 mL of a 1% (w/w) protein solution was added into the equipment glass column (40 mm prism), avoiding the formation of foam during addition. Internal gas air was sparged at a 0.3-L/m flow rate from the bottom of the column through a 16-40 µm filter (50 mm diameter) at room temperature (the total volume of the gas sparged was 100 mL). A camera and an infrared illumination source (λ = 850 nm) were positioned at a 60 mm height to record the foam structure and breakdown of the foam. Maximum foam height (mL), bubble count (mm^2) and R_{32} representing the bubble size (µm) were recorded through a 1-h run.

5.3.4. Statistical analysis

Data analysis was performed using Minitab® Statistical Software (LLC). A one-way analysis of variance (ANOVA) with Tukey's post-hoc test was run to find significant differences among all samples. To determine existing correlations between surface properties and functional properties, a Pearson product-moment correlation test was performed. A two-way ANOVA was performed to investigate the interaction between effects (pH and temperature) for all the determinations. The pH shifting treatments were run in triplicates, and all determinations were determined in duplicates (n=6). Results are shown as the mean ± one standard deviation.

5.4. Results and discussion

5.4.1. Surface properties

5.4.1.1. Surface charge

Results for the zeta potential of pH-shifted samples evaluated at pH 7 are shown in Table 5.1. Zeta potential values ranged between ~-22 to -28 mV. A two-way ANOVA showed that pH (p<0.001), temperature (p<0.05) and their interaction (p<0.01) were significant factors in the zeta potential of the pH-shifted samples. When the pH was shifted without heating, no major differences were found between the pH_2 (-23.5 mV), pH_7 (-24.63 mV) and pH_{10} (-24.85 mV) samples. However, when pH shifting was combined with heating, pH_{7heat} showed a net increase in the zeta potential values (p<0.001), indicating changes in the surface of the proteins charged groups due to the unfolding and refolding of the proteins due to the application of heat. However, pH_{2heat} and pH_{10heat} samples showed no major differences when compared to the non-heat-treated ones. The observed changes are hypothesized be attributed to the pH-shifted and heated samples (pH_{2heat} and pH_{10heat}) undergoing further unfolding due to the combination of treatments and consequently

increasing the protein-protein interactions leading to aggregation, which ultimately resulted in lower zeta potential values compared to the pH_{7heat} sample which was only exposed to heat treatment. Zhao *et al.* (2023) studied a pH shifting treatment on pea protein isolate and found similar zeta potential values ranging from ~25 to -35 mV measured at pH 7. Their findings showed a decrease in the zeta potential values when pH treatment was applied, and noted enhanced protein stability in solution, attributed to repulsion between the negatively charged proteins.

5.4.1.1.1. Surface hydrophobicity

The protein folding process is driven by the hydrophobic effect, burying hydrophobic groups towards the inside of the folded protein structure. However, some hydrophobic moieties remain exposed on the surface (McClements & Grossmann, 2021). The surface hydrophobicity of a protein indicates the presence of exposed hydrophobic patches on the surface of the protein. In the present study, it evidences the changes in conformation that the proteins can undergo due to pH shifting. These results can be found in Table 5.1. A two-way ANOVA showed that pH ($p< 0.001$), temperature ($p<0.001$) as well as their interaction ($p<0.001$) were found to be significant factors in the surface hydrophobicity of the evaluated samples. Results showed that the pH_{10} shifted proteins presented higher surface hydrophobicity compared to the pH_7 and pH_2 samples, indicating more unfolding and opening of the protein structure for the alkaline-shifted samples. Zhao *et al.* (2023) and Chang *et al.* (2023) found similar results for alkaline pH-shifted pea proteins and pea vicilin, respectively.

For the samples pH_{2heat} and pH_{7heat} it was observed that the heat treatment resulted in a greater increase in surface hydrophobicity, indicating that the application of heat further unfolded the protein structure. Sun *et al.* (2023) found similar results and reported a synergistic effect between pH shifting and mild heat treatment in the resulting surface hydrophobicity of soy protein isolates, suggesting changes in the protein conformation and tertiary structure of the protein. Nevertheless, for the pH 10 samples, the pH_{10heat} samples had a significantly lower hydrophobicity than the pH_{10}. This finding could be attributed to increased levels of protein unfolding and increased contact of non-polar groups towards the surface.

Table 5.1. Surface properties and functionality at pH 7 of pH-shifted and heat-treated samples.

Sample	Zeta potential (mV)	Surface Hydrophobicity (a.u)	Solubility (%)	FC (%)	FS (%)	EAI (m^2/g)	ESI (min)	ES (%)	Droplet size D[3,2] (µm)	Max Foam Height DFA (mL)
pH_2	-23.50 ± 1.97 ^{ab}	142.64 ± 14.64 ^{e}	16.80 ± 1.41 ^{d}	188.9 ± 13.1 ^{d}	88.8 ± 1.3 ^{a}	22.36 ± 0.58 ^{bc}	10.90 ± 0.13 ^{a}	93.0 ± 1.1 ^{bc}	9.65 ± 0.48 ^{ab}	120.28 ± 3.09 ^{ab}
$pH_{2\ heat}$	-22.26 ± 0.96 ^{a}	213.08 ± 18.48 ^{c}	16.25 ± 0.28 ^{d}	318.9 ± 19.1 ^{a}	87.6 ± 6.5 ^{a}	25.86 ± 1.08 ^{a}	10.78 ± 0.31 ^{ab}	92.7 ± 1.0 ^{c}	9.85 ± 0.26 ^{a}	117.53 ± 4.99 ^{b}
pH_7	-24.63 ± 2.07 ^{ab}	177.78 ± 11.50 ^{d}	38.51 ± 1.39 ^{c}	191.1 ± 10.0 ^{d}	91.3 ± 1.9 ^{a}	22.02 ± 1.74 ^{bc}	10.84 ± 0.35 ^{a}	93.3 ± 1.0 ^{bc}	9.96 ± 0.75 ^{a}	118.42 ± 5.86 ^{ab}
$pH_{7\ heat}$	-28.61 ± 2.48 ^{c}	239.39 ± 20.95 ^{bc}	53.14 ± 5.15 ^{b}	247.8 ± 16.6 ^{c}	44.1 ± 4.4 ^{b}	21.48 ± 0.99 ^{c}	10.38 ± 0.30 ^{b}	94.7 ± 1.0 ^{ab}	9.10 ± 0.06 ^{bc}	120.68 ± 3.86 ^{ab}
pH_{10}	-24.85 ± 1.75 ^{ab}	285.71 ± 19.00 ^{a}	51.27 ± 1.07 ^{b}	275.0 ± 16.6 ^{b}	88.4 ± 3.1 ^{a}	23.91 ± 1.39 ^{ab}	10.76 ± 0.07 ^{ab}	95.7 ± 0.8 ^{a}	9.06 ± 0.15 ^{c}	124.97 ± 3.48 ^{a}
$pH_{10\ heat}$	-26.31 ± 1.62 ^{bc}	246.16 ± 24.69 ^{b}	74.13 ± 4.89 ^{a}	235.6 ± 6.9 ^{c}	32.5 ± 2.6 ^{c}	25.03 ± 0.99 ^{a}	10.77 ± 0.14 ^{ab}	95.3 ± 1.0 ^{a}	8.40 ± 0.41 ^{c}	119.65 ± 3.30 ^{ab}

FC, Foaming capacity; FS, Foaming stability; EAI, Emulsion activity index; ESI, Emulsion stability index; DFA, Dynamic Foam analyzer.

Means within columns with different lowercase letters indicate significant statistical differences (p<0.05)

This exposure may have promoted protein aggregation potentially leading to a reduction in the surface hydrophobicity of the pH_{10heat} proteins. In summary, the determination of surface hydrophobicity suggests that pH shift treatments and in combination with temperature treatment led to structural protein changes. These changes resulted in alterations of the protein's surface properties influencing the protein's functional properties. The impact of surface hydrophobicity changes on protein functionality will be discussed in the protein functionality section.

5.4.2. Protein Functionality

5.4.2.1. Solubility

Protein solubility results determined at pH 7 are shown in Table 5.1. An analysis of the results showed that pH (p<0.001), temperature (p<0.001) and their interaction (p<0.001) had significant effect on the solubility of the resulting treated samples. Shifting the pH from 7 to 10 significantly improved the solubility of the pea proteins from 38.5 to 51.3%. However, samples shifted at acidic pH, both heat and unheated, showed a significant decrease in the protein solubility of the samples at ~ 16%. Jiang *et al.* (2009) proposed that the increase in solubility of pH_{10}-shifted proteins could be attributed to stronger hydrogen bonds between water and negatively charged proteins. Jiang *et al.* (2010) proposed that enhanced solubility for alkaline-treated proteins can also be attributed to the disruption of protein subunits in soy glycinin, by the disruption of disulfide bonds that hold together the 11*S* protein, consequently increasing the solubility. Jiang *et al.* (2009) found that subjecting the samples to extreme pHs (1.5 and 12) resulted in a reduction in solubility, aligning with the findings in the current study where samples treated at pH_2 experienced a decrease in solubility. These more severe conditions may suggest an increase in protein unfolding leading to aggregation of proteins via hydrophobic interactions, leading to an overall decrease in the solubility of the samples. Jiang *et al.* (2010) reported that pH shifting to acidic pHs can lead to cross-linking of glycinin, also contributing to decreased changes in solubility. Heating at 80ºC for 1 h, led to increased solubility for the pH_7 (from 38.5 to 53.1%) and pH_{10} (from 51.3 to 74.1%) samples. Wang *et al.* (2018) subjected hemp proteins to alkaline pH shifting combined with heating at 80ºC for 1h and found that the pH-temperature combination increased the solubility from ~25% to ~97%. Sun *et al.* (2023) found similar results proposing a synergistic effect between heat and alkaline pH shifting treatment, where mild heating (50ºC) and increasing pH alkaline treatments (pH 7 to 12) improved the solubility of a soy protein isolate. In conclusion, applying heat to both

the pH_7 and pH_{10} samples resulted in increased solubility. Additionally, heating these samples further improved solubility. However, acidic pH shifting as well as its combination with temperature, negatively impacted the solubility of the samples.

5.4.2.2. Emulsifying properties

The emulsifying properties of the pH-shifted samples are presented in Table 5.1. An analysis of variance showed that pH (p<0.001), temperature (p<0.01) and the interaction between pH and temperature (p<0.01) were significant factors for EAI of pH-shifted proteins. In the absence of heat, EAI values were all similar regardless of the pH treatment ranging between 22.0 to 23.9 m^2/g. When heating was applied, EAI values for pH_{2Heat} and pH_{10Heat} increased similarly (~25.5 m^2/g) while values remained lowest for pH_{7Heat} (21.5 m^2/g). Possibly the higher charge on the protein at pH_{7Heat} led to greater electrostatic repulsion at the oil-water interface during formation of the viscoelastic film leading to reduced emulsion formation relative to the other sample treatments. Overall, EAI values increased slightly from ~22 m^2/g to 25.9 m^2/g with the addition of heat, regardless of the pH. Wang *et al.* (2018) observed that a pH shift treatment to pH 12 did not lead to a significant rise in EAI for hemp seed proteins, however, when the pH shifting treatment was combined with heating at 60 ºC a significant increase in EAI was observed from ~5.4 to ~7.4 m^2/g. Additionally, the authors also found that an increase in emulsifying activity corresponded with an increase in surface hydrophobicity. This shows that an increase in hydrophobic groups, due to protein unfolding, contributed to an increased surface activity of the proteins at the oil/water interface. Similar outcomes are shown in the present study, where pH_{10heat} and pH_{2heat} also presented significantly higher surface hydrophobicity results compared to the control, evidencing protein unfolding and increased hydrophobic groups towards the protein's surface. In agreement with these results, a positive correlation between emulsion stability and surface hydrophobicity was found in the present study (r=0.628, p<0.001). Results of the ESI are shown in Table 1, where no major differences were observed between treatments (ranging between 10.4-10.9 min). Findings showed that in dilute emulsions, although changes in emulsion forming properties (*e.g.*, EAI) were observed (pH_{7Heat}), this did not alter the stability of those emulsions once formed.

An analysis of variance showed that only pH (p<0.001), was a significant factor for ES of pH-shifted proteins; although changes were not that substantial. Note, ES experiments were performed by creaming using a more concentrated solution (1%, w/w) than used in the EAI/ESI

experiments (0.25%, w/w). ES for pH-shifted samples without heat showed ES to be similar for pH_2 and pH_7 (~93%), then increased to (~96%) at pH_{10} (Table 5.1). For the samples with heat treatment, ES also increased when the pH was shifted: at pH_{2Heat} ES was ~93% and increased to ~95% at pH_{7Heat}/pH_{10Heat} (Table 5.1). Overall, ES increased slightly from ~ 92 to 96% with the addition of heat, regardless of pH, possibly explained by the increased solubility of these heat-treated proteins (Table 5.1). Further, overall droplet size ($D_{3,2}$) was slightly smaller with heat-treated samples, reducing from ~ 9.1 μm to 8.4 μm which aided in improving emulsion stability in the present study (Table 5.1). To achieve high emulsifying properties, solubility is usually a prerequisite since the proteins need to solubilize to be able to migrate to the interface and reduce the interfacial tension Jiang *et al.* (2018). A positive correlation was observed between ES and solubility (r=0.73; p<0.001) in the present study. Tang *et al.* (2023) also found a positive correlation between ES and solubility for pH-shifted proteins, particularly for pH10-treated oat proteins with increased solubility and ES (~90%) when compared to both the untreated and acid pH-shifted proteins (~29% for both samples). Furthermore, pH shifting, due to partial unfolding of the proteins, increases the flexibility of the proteins, allowing them to better accommodate at the interface, ultimately increasing the stability of the emulsions (Shen *et al.*, 2022; Wang *et al.*, 2018). In the present study, a positive correlation was found for ES and surface hydrophobicity (r = 0.628, p<0.001) where possibly the partial unfolding led to increased interaction of hydrophobic groups at the interface and among proteins surrounding the oil droplets, increasing the stability of the emulsions. In addition, a negative correlation was observed between ES and zeta potential (p<0.05, r = -0.387) where the negative charges at the protein's surface at pH 7 led to repulsion between protein-coated oil droplets, leading to higher emulsion stability.

The droplet size distribution was measured immediately after the samples were homogenized. Bimodal distributed curves were obtained, and the surface-average droplet diameters ($D_{3,2}$) are shown in Table 5.1. All emulsions showed similar droplet size distribution curves, with the highest peak in the range of 10 to 100 μm, and a small curve around ~ 0.4 – 6 μm. An analysis of the $D_{3,2}$ data showed that pH (p<0.001), temperature (p<0.01) and their interaction (p<0.05) were significant factors in the droplet size of the prepared emulsions from the pH-shifted samples. The untreated samples showed higher droplet sizes, however, when the pH was shifted, the droplet sizes decreased. In addition, droplet sizes decreased further when the pH was shifted in

combination with heat treatment. Jiang *et al.* (2014) reported smaller droplet sizes for alkaline pH-treated samples ($D_{3,2}$ ~1.5 μm) compared to native pea proteins ($D_{3,2}$ ~2.3 μm).
The smallest droplet sizes were found for the pH_{10} and pH_{10heat} samples. These results align with ES results, and a negative correlation was found between ES and droplet size (p<0.001, r= -0.61) where samples with smaller droplets showed a slower creaming rate, resulting more effective against emulsion instability.

5.4.2.3. Foaming properties

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

The foaming capacity and stability of the treated samples are shown in Table 5.1. Changes in pH (p<0.001), temperature (p<0.001), and their interaction were found to be significant factors in the FC of the samples. Shifting the pH of the samples with no heating, showed an increase in FC for the pH_{10}, increasing from 191% for the untreated sample to 275% for the alkaline shifted sample. However, an acidic pH shifting decreased the FC to 189%, lower than the untreated sample, possibly due to the aggregation of proteins and decreased adsorption of the proteins to the interface (Figueroa-González *et al.*, 2022). When heating was applied, however, there was an improvement in the FC for the pH_{7heat} sample, as well as for the pH_{2heat} sample which exhibited the highest FC at 319%. Tang *et al.* (2023) also reported a higher increase in the FC of rice proteins when the proteins were shifted and heated at pH 2 compared to the shifted and heated proteins tested at pH 10. An alkaline shifting and heating of the sample showed that pH_{10heat} sample only improved slightly (235%) compared to the untreated sample. The improvement of FC in the different tested samples can be related to increased surface hydrophobicity's of the samples as well as increased flexibility of the proteins (Tang *et al.*, 2023; Wang *et al.*, 2023). A Pearson's correlation analysis showed that FC and surface hydrophobicity had a positive correlation (r = 0.534, p<0.01), which could explain the above results, where the pH_{7heat} and pH_{2heat} samples exhibited higher surface hydrophobicity, allowing stronger protein-protein interactions as well as protein-interface interactions. Alavi *et al.* (2021) also found an increase in the foam volume of faba bean proteins and an increase in surface hydrophobicity of samples shifted at pH 11 (269%) as well as combined with heat treatment (376%) compared to pH 7-unheated samples (122%).

Regarding the stability of the prepared foams, pH (p<0.001), temperature (p<0.001) as well as their interaction (p<0.001) were significant factors in the stability of the foams. When the pH of

the samples was shifted to either pH_2 or pH_{10}, the stability of the foams ranged from 91.28% for the untreated sample, to 88.4% for the alkaline shifted sample and ~ 89% for the acidic shifted sample, but overall, there were no differences in their stability. On the contrary, when the samples were heated (pH_{7heat}, ~44%) and pH shifted to alkalinity and heated (pH_{10heat}, ~32%) their stability decreased significantly. Tang *et al.* (2023) reported no increase in the FS for the pH shifted nor for the pH shifted and heat-treated samples, as well as reporting a negative correlation between solubility and FS, explaining that lower solubilities could produce stronger and more resistant films surrounding the air bubbles, resulting in increased stability of the foams. In the present study, pH_{10heat}, and pH_{7heat} which presented the lowest FS also showed increased protein solubility. The same negative correlation between solubility and FS was found in the present study ($r= -0.76$, $p< 0.001$). Other studies reported no changes in the stability of the prepared foams for acidic and alkaline pH shifted chickpea proteins, but an overall increase in the FC of the samples (Wang *et al.*, 2022), while Chang *et al.* (2023) reported no differences in the FS of pea vicilins pH shifted (pH 12) and heat treated compared to the control, but an increase in the FC of these modified proteins. In conclusion, applying a pH treatment and in combination with heat led to an overall increase in foam volume, but lower foam stability.

5.4.2.3.2. Dynamic foam analysis

Observation of the formation and breakdown of bubbles over time was determined for the prepared samples using a dynamic foam analyzer. Note, foams were produced using a sparging method versus the aforementioned section which used a high-shear mixer. The maximum foam height achieved was recorded (Table 5.1). Overall, no differences were found between samples, where foam heights ranged between ~125 – 118 mL. The difference in foam height between samples for both methods can be related to the foam generation method. Sparging air through a porous disc achieves foams with characteristics that are directly influenced by different factors such as the disc pore dimension, pore distribution and gas flow, while foaming achieved using a homogenizer achieves foams that are dependent on the input of the mechanical energy, including speed and homogenization time (Drenckhan & Saint-Jalmes, 2015).The sparging method is hypothesized to have produced more homogeneous foams due to the uniformity of the pores through which the air passed, leading to foams with similar height. On the contrary, the high shear method achieved bubbles with more dispersed sizes impacting the foaming capacity.

Results from the bubble count and the Sauter mean radius (R_{32}) representing an average of the droplet sizes, monitored at the beginning and at the end of a 3600 s run are shown in Fig. 5.1 and 5.2. Results showed that as time progressed, the air bubbles burst and formed bigger-sized bubbles, evidenced by an increase in the bubble size of the foams and a decrease in bubble count. Further, a negative correlation was found between the Sauter mean radius ($R_{3,2}$) and bubble count both at time zero (time zero was considered as t=40 s, which was the time for foam stabilization) and at the end of the run (t=3600 s) (time cero: r= -0.94, p<0.001, time 3600s: r= -0.716, p<0.001). Figure 5.3 shows the bubble structure and size over a 3600 s period. The DFA software shows air bubbles of smaller sizes coloured in green, and as these increase in their size they are shown in blue, purple and pink colours. Sample pH_{2heat} (Fig 5.1 and 5.2) shows that the change in bubble sizes ($R_{3,2}$) at the beginning and end of the analysis showed the biggest difference, compared to other samples. Air bubbles coalescing and growing into bigger bubbles can be explained by the lower value of surface hydrophobicity for sample pH_{2heat} where less hydrophobic groups at the surface of the protein could be related to less interaction of the proteins towards the air interface, creating more instability of the foam.

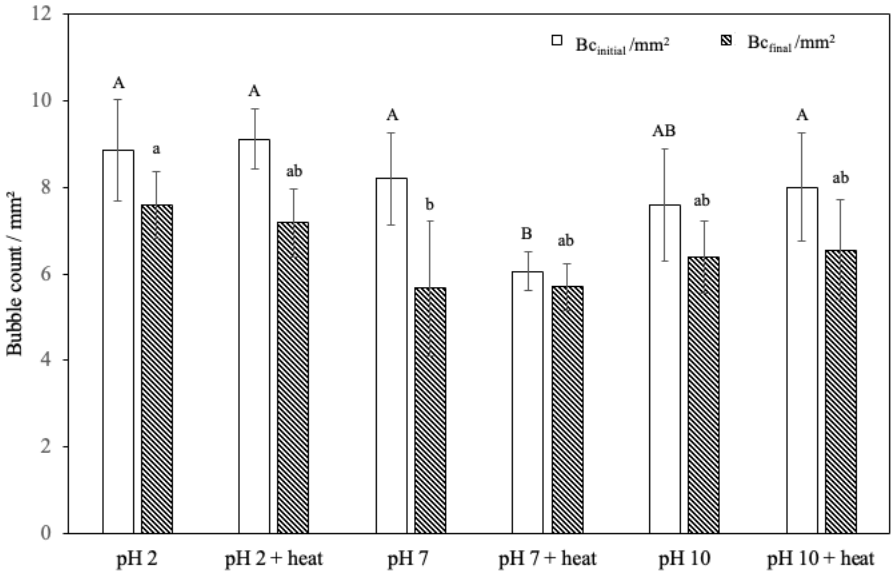

Figure 5.1. Bubble count per mm^2 for foams prepared using a dynamic foam analyzer. The same capital letter represents no significant differences across the initial bubble count ($Bc_{initial}$/mm^2, p<0.05)). The same lower case letters represent no significant differences across the final bubble count (Bc_{final}/ mm^2 , p<0.05).

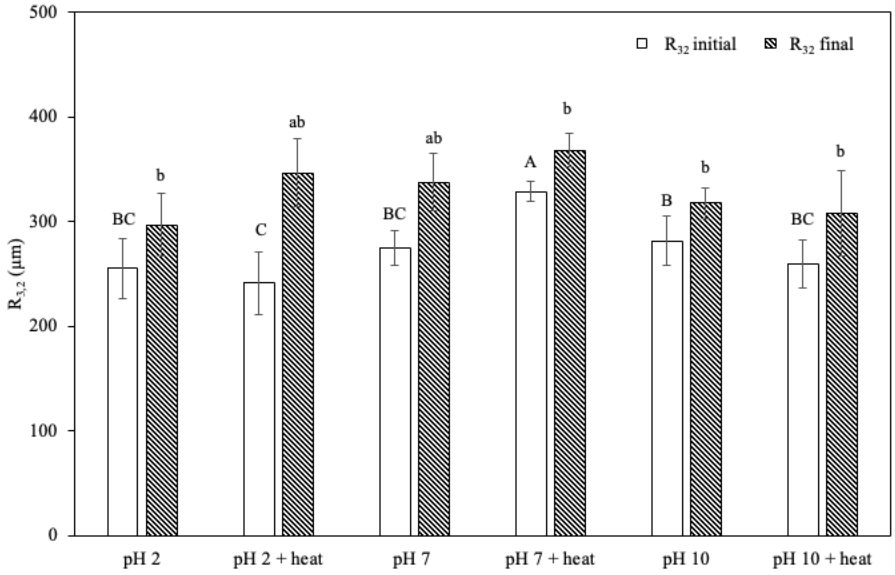

Figure 5.2. Sauter mean radius ($R_{3,2}$, µm) of air bubbles from foams prepared using a dynamic foam analyzer. The same capital letter represents no significant differences across the initial bubble radius ($R_{3,2}$, p<0.05). The same lowercase letters represent no significant differences across the final radius ($R_{3,2}$, p<0.05).

An analysis of the parameters showed that the foams produced with the pH_{7heat} treated samples showed the biggest initial and final bubble sizes ($R_{3,2}$) compared to all the other samples (Fig 5.2 and 5.3). The initial Sauter mean radius was found to positively correlate with solubility (r = 0.365, p<0.05), where a higher solubility of the proteins allowed to faster migration to the interface, however also possibly a less stronger interfacial film leading to bubble coalescence (Amagliani *et al.*, 2021) and larger air bubbles.

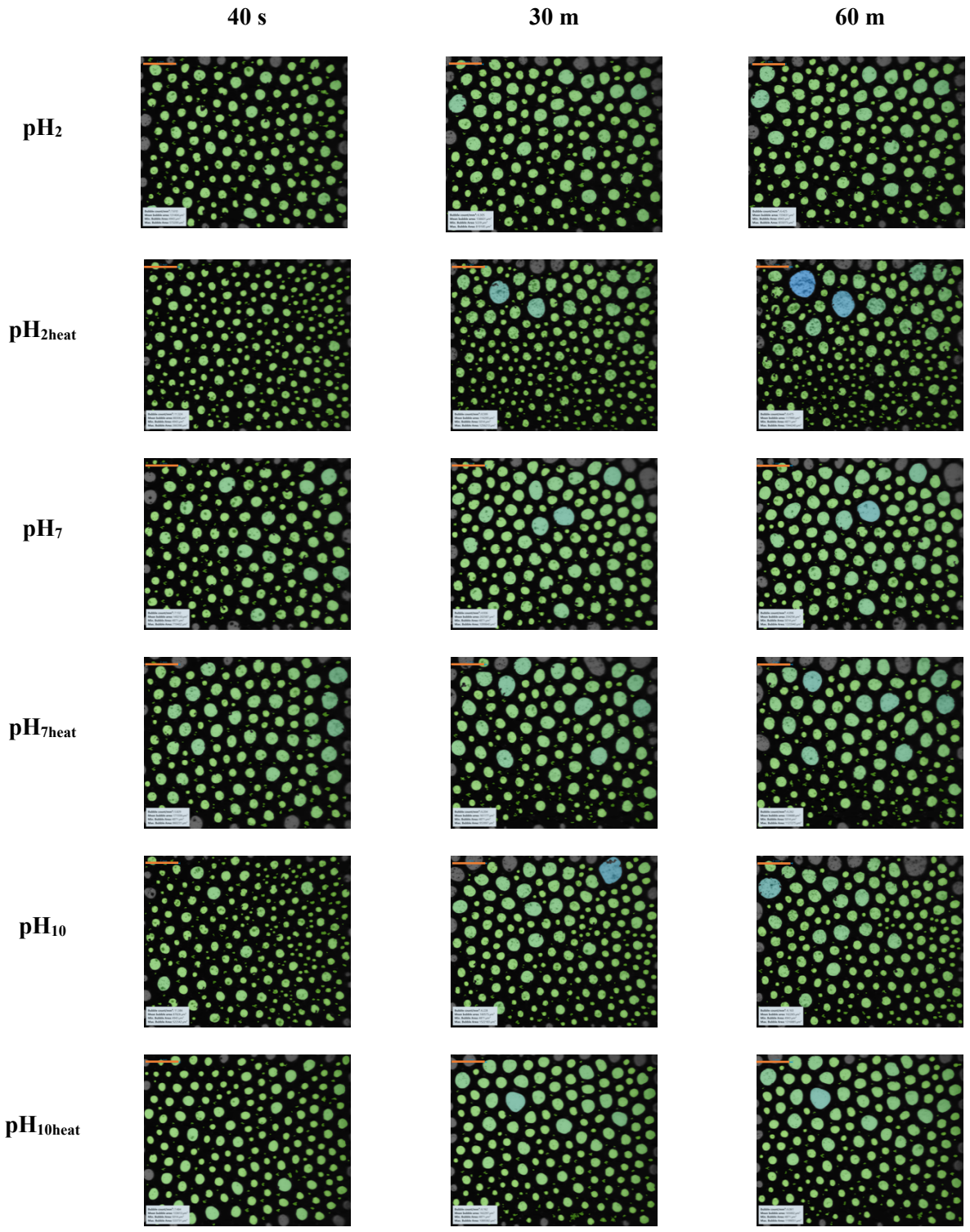

Figure 5.3. Bubble structure evolution for foams during a 1 h run using a dynamic foam analyzer. The red line in the pictures represents a 2 mm length.

5.5. Conclusions

The present study explored the effect of a pH-shifting treatment on the surface and functional properties of a commercial pea protein isolate. The results showed that applying an alkaline pH shifting, or pH shifting accompanied by heating, enhanced the solubility of the commercial pea protein isolate, while an acidic treatment significantly reduced the solubility. pH shifting improved some functional properties like foaming capacity and emulsion stability. However, a neutral or negative effect was found on other properties like foaming stability. For example, alkaline pH-shifted proteins could be used in beverage applications, while heated and acidic pH-shifted proteins could be used to facilitate the formation of foam in aerated products. In conclusion, the use of pH shifting to modify pea protein functionality can be a useful method for tailoring the use of these proteins in different food products.

6. GENERAL DISCUSSION

The overall goal of this research was to examine the effect of protein extraction factors (extraction method, f:w ratio used in extraction and particle size of the pulse flours) as well as a protein modification strategy (pH-shifting) on the resulting surface and functional characteristics of plant proteins. The findings of these studies will enable us to better understand pulse proteins and their functionality, which is crucial for understanding how proteins will behave when applied to food products. This can benefit the development of products with high protein content to meet consumer demands. In addition, the insights gained from these studies can contribute to a deeper understanding of how pulse proteins can be tailored through their processing, including extraction methods, extraction conditions and protein modification strategies. The tailoring of proteins allows for the obtention of ingredients that meet a desired functionality, such as enhanced solubility, foaming or water holding capacity. These customized proteins can be used in different applications, broadening the use of pulse protein ingredients and pulse consumption. The increase in the use of pulse protein ingredients actively contributes to the growth, versatility, and popularity of pulse-based products.

The first study (Chapter 3) explored two factors influencing the alkaline extraction–isoelectric precipitation (AEIP) process of protein extraction: a) the impact of using reduced amounts of water during extraction (compared to the commonly used 1:10 f:w ratio) and b) the influence of the particle size of flours from yellow peas, green lentils, kabuli chickpeas and navy beans. Furthermore, not only processing-related parameters were studied, like extraction and protein yields, but also the impact of these factors on the functionality of the resulting isolated proteins. The flours used in this study were milled using a Ferkar mill and sieved through two mesh sizes: 0.5 mm (P1) and 1.27 mm (P2). To test the impact of water reduction, P1 flour was used. Results showed that variations in the f:w ratios (1:10, 1:7 and 1:5) did not show differences in the extraction yields. However, a reduction in water significantly influenced the protein yield (amount of protein recovered compared to the initial amount of protein) for all the pulse types evaluated. For example, navy beans showed a reduction of ~8% protein yield when reducing the f:w ratio

from 1:10 to 1:5, while yellow peas, green lentils and chickpeas showed decreases of ~7, 4.5 and 4.7%. Mass principle and difference of concentration gradients as driving forces of this process apply to these results, where greater differences in the concentration gradients for higher f:w ratios led to increased results (Pinelo *et al.*, 2005). Relating to the protein content, a reduction in water is desired to make the process more cost-effective and sustainable; however, a reduction in water does have an impact on the amount of protein extracted, and this can lead to a negative economic impact since the unextracted proteins will be discarded along with the starch fraction.

Based on the obtained results and to explore the effect of particle size on protein extraction, extractions with coarser particle sizes (P2) were carried out at a 1:10 f:w ratio. Results showed that a change in the particle size of the starting flours affected extraction and protein yields, where finer flours achieved increases of 0.4 to 3.2% for protein isolate recovery and an increase of 7.8 to 12.2% for the recovery of proteins. Furthermore, the protein contents in the extracted proteins were higher for the isolates extracted with P1. This difference in protein content with the change in particle size of the flour varied depending on the pulse type and ranged from ~7.9% for chickpeas to 10.9% for navy beans. These results highlight the importance of the milling process and control of particle size before extraction since using finer flours can positively impact the process yields.

Based on the protein composition of the resulting isolates, this study showed differences in the L/V ratios between the different pulse varieties, and they were found in the following order: CH > YP > GL > NB. These differences in protein composition impact the functionality of the proteins since the difference in their protein structure and size impacts how these proteins interact with each other and at interfaces. In terms of the effect of water use on the extraction, no changes were observed in the composition of proteins extracted. Different particle sizes didn't impact the protein composition; only differences were found for CH, exhibiting a higher L/V ratio for P2 than P1. Functionality results showed no differences with water reduction. However, there were differences in functionality depending on the pulse variety. For example, solubility was the lowest for GL (~53%), while it was highest for NB (~87%). These differences were attributed to protein composition since NB had a low L/V ratio, suggesting that high vicilin content, rich in aspartic and glutamic acid, allowed more protein-water interactions. Different particle-sized flours in protein extraction showed some differences in functionality but not for all pulses. For example, YP extracted from P1 flours resulted in higher zeta potential, solubility and FC than the proteins extracted from P1 flours. Possibly, extractions from smaller particle-sized flours resulted in a more

efficient extraction and dissociation of subunits of the proteins due to increased surface area, leading to changes in functionality.

In conclusion, results from this study show that controlling the particle size of the pulse flours can aid in protein isolate production through AEIP.

In the second study (Chapter 4), following the findings of the previous research on which f:w ratio (1:10) and particle size flour (P1) achieved the best results, the effect of two different extraction methods (AEIP and SE) on the surface, functional and nutritional properties were investigated. Results showed that the extraction method had a significant factor on the process, where the extraction yields were higher for SE-extracted proteins. However, the protein yields obtained through AEIP were higher for CH and GL, while YP and NB obtained higher yields for the SE method. The results suggest that the SE method could be more profitable for the extraction process depending on the pulse type. However, modifications can be made to the scale-up process of the extractions performed in this study. For instance, the SE method used dialysis to remove salts, but this process is slow since the exchange of salts through the porous membrane is not forced, and it requires large amounts of water to use the difference in concentration as the driving force of the salt exchange. A faster option could be ultrafiltration, which requires a pump to recirculate the extracted proteins and salt through a porous column, achieving similar results by separating the proteins in the filtrate and the salts in the retentate. Another modification to introduce to both processes is the drying method. The current study employed freeze drying, a preferred method for maintaining the native structure of proteins, as it eliminates the need for heat during the drying process. In this technique, water is extracted from a solid frozen state to a gas state (lyophilized), ensuring protein integrity. However, this method is costly on a small scale and not economically viable at larger scales. A more cost-effective alternative is spray drying. However, it involves heat application, potentially leading to protein denaturation.

The protein composition of the protein isolates was analyzed using SDS-gel electrophoresis. The main findings showed that the SE extraction method achieved higher A/G ratios, indicating that the SE method was more efficient at extracting a higher albumin content and a mixture of albumins and globulins, while the AEIP mainly extracted globulins at their isoelectric point. These differences in the extraction of different proteins impacted protein functionality. The SE samples showed higher solubilities, suggesting that the presence of albumins, which are smaller proteins and water-soluble, contributed to the increase of this functional property. Furthermore, high

solubility combined with higher albumin proteins, which are more flexible than globulins, increased other functional properties, such as emulsion activity (EAI) and foaming capacity (FC).

Exploring the relationship between sample extraction methods and the consequential impact on both protein composition and its relationship with protein functionality, showed that the method of extraction not only influences on protein recovery, but also can influence on the presence of other components such as soluble fiber, presence of carbohydrates, phenolics, among others. These components can also impact on how proteins interact with each other, with water and at interfaces, in consequence altering their functionality.

The amino acid composition of the extracted proteins showed that the common limiting amino acids in pulses were methionine, cysteine, and tryptophan. The extraction method also influenced the phenolic content in the final protein isolates. The SE method achieved a higher extraction of phenolics than the AEIP method, which could be mainly attributed to the higher affinity of phenolics towards albumin proteins (Sęczyk *et al.*, 2019). A higher phenolic content also impacted the nutritional properties of the protein isolates, where the SE samples showed lower IVPD /IVPDCAAS compared to the AEIP proteins. The presence of phenolic compounds and their interaction with proteins could impact the bioavailability of the proteins when consumed. Besides the presence of phenolics, the AEIP samples could also have exhibited a higher IVPD due to greater denaturation of the proteins during the extraction process, leading to a more open structure and, therefore, higher accessibility of enzymes.

In conclusion, studies 1 (Chapter 3) and 2 (Chapter 4) contributed to a better understanding of the impact of extraction conditions and methods on the protein extraction of pulses. The extraction method, in particular to the second study, impacted the type of proteins being extracted, consequently influencing the protein isolates' functionality and nutritional properties.

The third study (Chapter 5) explored how to improve the functionality of a commercial pea protein isolate. Plant proteins exhibit good functionality. However, their uses as ingredients in food products can be limited due to their large size and low flexibility, leading to low solubilities and lower performance as emulsifying or foaming agents. This last study aimed to use a pH-shifting method to improve pea protein functionality further using a relatively simple modification process that involves subjecting a protein solution to a change of pH, leading to the unfolding of the protein structures. When the proteins unfold, the hydrophobic groups that were previously buried became exposed to the surface of the protein. When the pH of the proteins was re-adjusted to neutrality,

the protein refolded to a structure different than the initial one. This new structure, due to higher surface hydrophobicity, was expected to increase the surface properties of the proteins compared to an untreated control. A set of samples was also subject to pH shifting and temperature to evaluate the effect of the combination of both treatments on the resulting functionality.

The results on surface properties indicated that there were no major changes in surface charge. However, there were changes in surface hydrophobicity that indicated changes in the tertiary structure of the proteins. Some samples showed an increase in this property, suggesting greater unfolding, such as samples shifted at pH 10 and, pH 10 with heat. However, acidic shifting led to lower hydrophobicity values than the control, possibly indicating extensive unfolding and protein aggregation. An alkaline pH shifting led to a significant increase in solubility, hypothesized due to the separation of protein subunits and changes in the quaternary structures of the globulin proteins. In contrast, an acidic shifting decreased the solubility by ~20%. Extensive protein unfolding at this extreme pH could be a possible hypothesis for this decrease, leading to increased hydrophobic interactions and protein aggregation. The changes in solubility and surface hydrophobicity also impacted the foaming and emulsifying properties of the modified proteins. A slight increase in emulsifying activity was evidenced when the samples were subject to shifting and heat. A positive correlation was found between emulsion stability and solubility and emulsion stability and surface hydrophobicity, where an increase in solubility allowed the proteins to migrate at the interface and an increase in surface hydrophobicity to increase the interactions at the oil-water interface, reducing the interfacial tension. The treatments also affected foaming properties, where an increase in surface hydrophobicity and increased flexibility of the proteins due to pH shifting led to increased interactions between proteins and with the interface, resulting in increased foaming capacity. Foaming stability results showed that samples with lower solubilities, like the acidic treated ones, led to increased stability of the foams due to the formation of stronger films surrounding the air bubbles, preventing their coalescence. An analysis of the foam using a dynamic foam analyzer is a novel approach to studying the foam structure, and allowed the monitoring of the bubble sizes and foam stability through time, and provided a better understanding of the role of proteins as stabilizing agents at interfaces.

In addition, it's worth mentioning the effect on pH shifting on the protein quality of the samples. An alkaline pH shifting treatment in combination with heat can lead to the formation of undesired compounds such as lysinoalanine or oxidation of cysteine residues that could overall

negatively impact on the availability of these amino acids (Zhang *et al*., 2018). In the present study, a pH of 10 was selected for the alkaline treatment, to avoid the formation of these compounds at more severe alkaline conditions.

In conclusion, the last study (Chapter 5) explored a protein modification method that resulted in protein structure changes that improved some functional properties. A pH shifting method is a relatively simple method that doesn't require the use of expensive equipment and only requires the addition of base/acid and the application of heat. This protein modification can add value to pea protein ingredients since the improvement in functionality, such as solubility, foaming capacity and emulsifying properties, can allow pea proteins to expand their uses and applications compared to untreated pea ingredients.

In summary, this work allowed us to draw some recommendations about extraction methods, applications and uses of pulse proteins:

- A finer flour (sieved through a 0.5 mm mesh size) is recommended for protein extraction since it will maximize the extraction and protein yields, allowing a more efficient extraction process without impacting on the functionality of the proteins.
- A reduction of water use during extraction is not recommended since a reduction in water negatively impacts the extraction of proteins, achieving lower protein yields. An analysis of the economic impact of using less water over decreased protein collection is needed to further assess this decision.
- In terms of common pulse protein applications:
    - An AEIP extraction method is recommended for YP, CH and NB as meat binders for their high WHC.
    - A SE extraction method is recommended for all pulses for their application as ingredients in baking applications due to the higher foaming properties. In particular, CH showed the highest foaming properties of all pulses and could be a good ingredient for aerated products such as mousses, confections, and dairy analogs.
    - For beverage applications where high solubilities are required to avoid precipitation of protein ingredients, AEIP-NB and SE-GL are recommended (solubilities of ~88 and 91%, respectively).
    - Overall, all pulse proteins tested showed good functionality, however, CH showed the less variation in solubility and other functional properties across the extraction methods

tested, making this pulse protein suitable to be extracted through different techniques obtaining a reliable consistent ingredient.

- Alkaline pH shifting is a recommended protein modification method for beverage applications, since it increases solubility and emulsifying properties. Conversely, an acidic pH shifting is not recommended for the same applications due to the negative impact in solubility. However, it could be applied in foaming applications due to the high stability of the foams formed.

Overall, this work contributes to a better understanding of wet protein extraction and how different parameters affect both yields and functionality of the extracted proteins. When used in food applications, proteins are expected to contribute to nutrition, to be versatile and perform well when used for their functional properties. One of the major challenges of proteins as ingredients is obtaining a protein source consistent in its functional attributes. Different factors can affect the protein composition of pulses (Mertens *et al.*, 2012), including cultivar, growing season, harvest, etc., over which the food industry can have little control. However, the extraction process (from milling the seeds to extraction and drying of the final product) is also a major source of variation, impacting the protein fractions extracted and the structure and function of the proteins. However, if known and studied, these factors can be controlled to obtain consistent ingredients with known functionality tailored to a desired target use. In addition, in the protein isolation and formulation of plant-based foods, other challenges also arise and are essential for the success of these products such as developing products rich in high-quality proteins and sensory attributes that resemble the products they are replacing. A holistic future work in this area could address aspects related to the application of pulse proteins in food products, addressing their nutritional properties, functionality and how the addition of the proteins impacts the aroma, flavor and textural attributes of plant-based products.

7. OVERALL CONCLUSIONS

The overarching goal of this research was to investigate three main objectives: to study the impact of extraction parameters on pulse protein extraction, to study different extraction methods and their impact on the resulting protein properties, and to study the modification of a pea protein isolate using a pH shifting method to improve its emulsifying and foaming properties. These studies were carried out using flours of two different particle sizes for four different pulses for studies 1 (Chapter 3) and 2 (Chapter 4), and a commercial pea protein isolate was used for protein modification in study 3 (Chapter 5). A commercial pea protein isolate was chosen for the last study since a large quantity of isolate was required to prepare the pH-shifted samples. An extraction of this protein would have required several extractions to obtain enough sample. Therefore, choosing a commercial pea protein was a practical approach to ensure enough protein isolate was available throughout the study.

The findings of the study of different extraction parameters showed an impact on the resulting protein functionality. In addressing the hypothesis that a reduction in water could lead to a decrease in protein yields, this study showed that a reduction of water results in protein losses in the extraction process. However, milling flours to smaller particle sizes could be a practical approach to increase the extraction yields during protein extraction without producing major effects on their resulting functionalities. In terms of the different pulses evaluated and their functionalities, there were differences between the evaluated pulses that made them suitable for various applications; the high solubility of navy beans demonstrates suitability for beverage applications, while chickpea proteins could be ideal for aerated products like desserts and mousses due to their high foaming capacities.

The study of SE and AEIP as extraction methods for protein isolation highlighted the difference in effectively extracting different proteins. These differences in protein extraction and the use of different pulse sources directly impacted the protein isolates' functionality and nutritional properties. These findings provide valuable insights for food processing industries and product developers, aiding in informed decisions regarding the extraction process and ingredient selection

tailored for a desired application. For instance, SE-yellow peas reported the lowest WHC (0.92 g/g), which wouldn't make them the best option to produce a meat analogue. In comparison, SE-GL (1.91 g/g) could be more suitable for this type of product that requires water retention to impart juiciness and act as a binder of ingredients.

The last section (Chapter 5) investigated the use of a pH shifting method, a relatively simple and accessible method, to enhance the foaming and emulsifying properties of a commercial pea protein isolate, which served as a model for pulse proteins. The findings of this study revealed that the enhancements in functionality were dependent on the pH shifting–temperature treatment, concluding that it could be a promising method for improving protein isolates' functional properties in food applications. The viability of implementing this method in the food industry considering costs and practicality could be assessed to determine its application.

Overall, this research explored different aspects of wet extraction of proteins and their impact on protein functionality. These results emphasize the importance of selecting the extraction method and conditions and the pulse species that allow better control over the resulting proteins, their functionality, and applications. This research contributes to the knowledge of plant-based proteins as functional ingredients for the food industry, which, through a better understanding of their extraction and their physicochemical and functional properties, will allow the tailoring and improvement of pulse protein products to produce reliable ingredients.

8. FUTURE STUDIES

This research provided a comprehensive study on pulse protein extraction, information on methods, extraction conditions, and protein functionality. Further exploration of the following aspects related to pulse proteins and their significance in the food industry as functional ingredients are warranted.

- Our study on protein extraction explored the use of reduced particle size on extraction yields, finding that finer flours increased the protein extracted in the process. However, reducing water was not feasible without losing proteins during the extraction. Further studies could address using finer flours combined with less water for extraction to examine how fine the flours should be to obtain the same yields as using a 1:10 f:w ratio but with reduced water use. To further explore the use of less water during extraction, the combination of protein extraction with microfiltration and the reuse of water could be explored, as well as a combination of dry extraction – wet extraction and water reuse/microfiltration.

- Different extraction methods impacted pulse protein isolates' surface, functional and nutritional properties. It would be interesting to investigate if the extraction methods (SE, AEIP) impact the sensory properties of the extracted isolates since the ultimate goal of applying proteins to food products is to make the food products acceptable to consumers. The extraction method could impact the aromatic profile and the presence of off-flavors in the protein isolates. This information could contribute to a better understanding of protein-aroma interactions during extraction and possibly avoid posterior de-flavouring strategies, which could further denature proteins and impact their functional properties.

- The present studies explored the proteins extracted using SDS-PAGE and surface hydrophobicity to indicate changes in the tertiary structures due to either extraction method or protein modification through pH shifting. Further exploration of protein structure could be

carried out through circular dichroism (CD) or Fourier transform infrared spectroscopy (FTIR) to investigate further changes in the secondary structures of the proteins.

- Protein modification, such as pH shifting, successfully improved some functionality aspects of pea proteins. The changes in protein structure could have an impact on protein digestibility, so it would be interesting to examine the nutritional properties of pH-shifted proteins to assess if these changes in structure led to increased digestibility due to increased flexibility and open structures of the proteins or if the accessibility of digestive enzymes was hindered by protein unfolding and refolding.

- With the prediction of protein structures and advances in computational tools that can aid in determining the native structure of the lowest energy a protein can take (Kuhlman & Bradley, 2019), it could be interesting to explore if the prediction of pulse protein functionality is possible from a theoretical point of view. However, in practice, the processes that the proteins are subject to during processing can alter these structures, and therefore, the functionality can also be impacted. In this case, it would be interesting to construct a database containing information on protein extraction and drying method, protein composition (L/V ratios, A/G ratios), protein structure (secondary and tertiary structures), surface properties (surface charge and surface hydrophobicity) as well as their associated functionality (foaming, emulsifying, gelling properties, etc.) to create a model that can ultimately predict the functionality of a protein based on the measurement of one or a few parameters.

- Using a dynamic foam analyzer allowed us to monitor the foam structure through time. Future studies could investigate the use of different protein concentrations on foam capacity and the foam life of pulse proteins. This information could provide a better understanding of foam stability and the creation of stable foam plant-based products, such as meringues, whipped preparations, plant-based creamers, and baked products.

- In addition, future research could involve applying and using proteins tailored through their extraction process and conditions to assess their performance when applied to the final products

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10. APPENDIX A: SUPPLEMENTARY MATERIAL FOR CHAPTER 4

Table A1. Results from Two-way Analysis of Variance (ANOVA) indicating the significance of the main factors (extraction method and pulse type) and their interaction on the physicochemical, functional, and nutritional properties evaluated.

Factors	Protein content	Ext. yield (%)	protein yield	TPC (mg gae / g	Zeta potential	Surface hydrophobi	Legumin/Vicilin ratio	Albumin /Globulin	Solubility (%)	EAI (m2/g)	ESI (min)	FC (%)	FS (%)	WHC (g/g)	OHC (g/g)	IVPD (%)	IVPDCAAS (%)
Main effects																	
Extraction method	NS	$p<0.001$	NS	$p<0.001$	$p<0.001$	$p<0.001$	NS	$p<0.001$	$p<0.001$	$p<0.001$	NS	$p<0.001$	$p<0.001$	$p<0.001$	$p<0.001$	$p<0.001$	$p<0.001$
Pulse type	$p<0.001$	$p<0.001$	NS	$p<0.001$	$p<0.001$	$p<0.001$	$p<0.001$	$p<0.001$	$p<0.001$	$p< 0.05$	NS	$p<0.001$	NS	$p<0.001$	$p<0.001$	$p<0.001$	$p<0.001$
Interaction																	
extraction method x pulse	$p<0.001$	NS	$p<0.001$	$p<0.001$	$p<0.001$	$p<0.001$	$p< 0.05$	$p< 0.05$	$p<0.001$	$p<0.001$	$p<0.05$	$p<0.001$	NS	$p<0.001$	$p<0.001$	NS	$p<0.001$

NS, Not significant $p>0.05$; EAI, emulsion activity index; ESI, emulsion stability index; FC, foaming capacity; FS, foaming stability; WHC, water hydration capacity; OHC, oil holding capacity; IVPD, *In-vitro* protein digestibility; IVPDCAAS, *In-vitro* protein digestibility corrected amino acid scores.